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14. ABSTRACT

Theprimarygoaloftheproposedworkistoidentifythegenesandtheirfunctionsinvolvedinmediatingtheanabolicresponsetomechanical stress.Duringthepastyear,wehaveusedstate-of-the-arttechnologiestoinvestigatetheroleofanumberofpotentialcandidategenesinmediatingskeletalanabolicresponsetomechanicalloading(ML).WefoundthatPTNdisruptioninmicecausedlessthantheanticipateddecreaseintheamountofnewboneformedinresponsetoMLinpartbecauseofcompensationbyincreasedexpressionofmidkine.ToinvestigatetheroleofRassignalingpathwayinML,weusedtransgenicmiceoverexpressingRassF1C,andfoundML-inducedboneformation(BF)wasincreasedintransgenicmice,thussuggestingaroleforRassignalinginanabolicresponsetoML.Based ontheestablishedimportanceofleptininregulatingBF,weevaluatedtheroleofleptinsignalinginmediatingMLeffectsonboneandfoundthatleptin-leptinreceptorsignalingmechanismmayplayakeyroleindeterminingthemechanosensitivityofosteoblasts.Webelievethatsuccessful accomplishmentoftheproposedstudieswillprovideabetterunderstandingofthemolecularmechanismsinvolvedinidentifyingthegenesandtheirfunctionasrelatedtomechanicalstress.

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I. Molecular Genetic Studies of Bone Mechanical Strain- In vitro studies

A. Introduction

Mechanical loading plays an important role in the maintenance of bone mass and strength [1-4]. Several reports have provided evidence that mechanical loading stimulates bone formation and that immobilization or a loss of mechanical stimulation, such as bed rest or space flight, leads to a decrease in bone formation and an increase in bone loss [5-7]. Recent studies in humans have demonstrated that bone anabolic response varies widely among individuals when subjected to the same degree of mechanical load ranging from good to moderate response [8-11]. Analogously, experimental animals, particularly inbred strains of mice, have also shown variability with respect to mechanical loading. Studies have shown that there are greater fold changes in bone marker genes as well as increase in the changes in bone parameters in C57BL/6J (B6) mice as compared with C3H/HeJ (C3H) mice when subjected to a same loading regimen [1, 12]. It is likely that these variations in the bone anabolic response, in both human and mouse models are largely, in part, regulated by genetic factors.

One of the approaches often used to study the genetic regulation of an observed phenotype is quantitative trait loci mapping. Using this approach, we have discovered several genetic loci that regulate bone adaptive response to loading [13, 14]. Thus, the finding of our studies provided evidence that bone formation response varies among individuals and is strongly regulated by genetic factors. Although quantitative trait loci analysis leads to a precise mapping of the genetic loci which contribute to our phenotype of interest, these regions are broad and contain many possible significant genes. The next phase of our study, therefore, lies in identifying which specific genes within our identified quantitative trait loci regions are associated with mechanical loading. In addition to this, we also performed genome-wide microarray analysis in a good responder C57BL/6J mouse, to identify possible candidate genes responsible for bone adaptive response to loading [15]. The results of this study have led to identification of thousands of genes that are differentially expressed in response to loading. Our next goal is to select potential candidates based on the microarray and quantitative trait loci findings, to study their role in anabolic effects of mechanical on bone formation.

B. Technical Objectives

1) Evaluate the role of pleiotrophin in mediating skeletal anabolic response to loading
2) Evaluate the role of Ras association domain family 1 isoform C (RassF1C) in mediating skeletal anabolic response to loading
3) Fate of newly formed bone in response to mechanical strain after cessation of loading
4) Generate Insulin like growth factor-I (IGF-I) conditional knockout mice for testing the role of locally produced IGF-I in mediating skeletal anabolic response to loading
5) Prepare annual progress report for submission to TATRC.

C. Body

1. <u>Heparin binding growth factor/Pleiotrophin</u>

Bone is a dynamic tissue that undergoes constant remodeling and turnover. These processes are mediated by two types of cells: osteoblasts, involved in bone formation, and osteoclasts, involved in bone resorption. The process of new bone formation can be classified into several stages. The pioneer stage refers to the recruitment of osteoblast precursor cells to a site for osteoid deposition. Upon arrival to the site, these precursor cells are then differentiated into fully functional osteoblasts. Bone formation has a high degree of specificity and must be precisely regulated, in terms of both the amount of formation and the site of formation which depends on the recruitment of bone forming cells. This process, therefore, is regulated by several osteoinductive factors, many of which are present within the bone matrix and have the potential to stimulate bone growth as well [16, 17]. One seemingly important factor among these is Pleiotrophin (PTN).

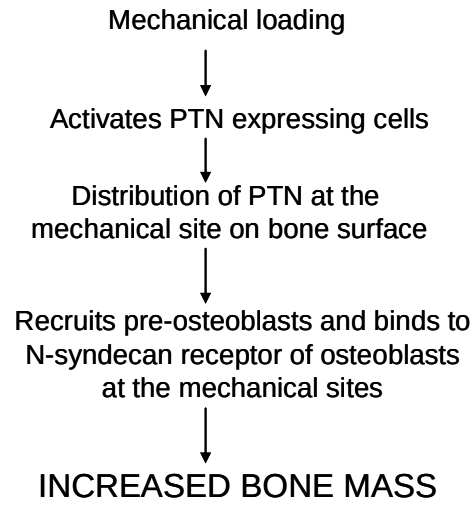

Figure 1: Model of Pleiotrophin (PTN) action in mediating skeletal anabolic effects to mechanical loading.

PTN, a 36 amino acid bone growth factor rich in lysine and cysteine residues, is also known as Osteoblast Specific Factor 1. PTN is involved in diverse functions, which includes: cell recruitment, cell attachment and proliferation, differentiation, angiogensis, and neurogenesis [18-20]. *In vitro* studies have demonstrated that PTN has the ability to promote adhesion, migration, expansion and differentiation of human osteoprogenitor and MC3T3-E1 cells [21-23]. *In vivo* studies using transgenic approach have shown that ovariectomy induced bone loss, due to estrogen deficiency, were protected by an increase in the expression of the PTN gene [24] transgenic study, with over expression of the human PTN gene showed an increase in cortical thickness, bone volume and cancellous bone volume [21]. In addition, immunocytochemistry studies has provided visual evidence for PTN at the site of new bone formation [21, 22]. Few other studies have shown that the cell surface receptor for PTN,

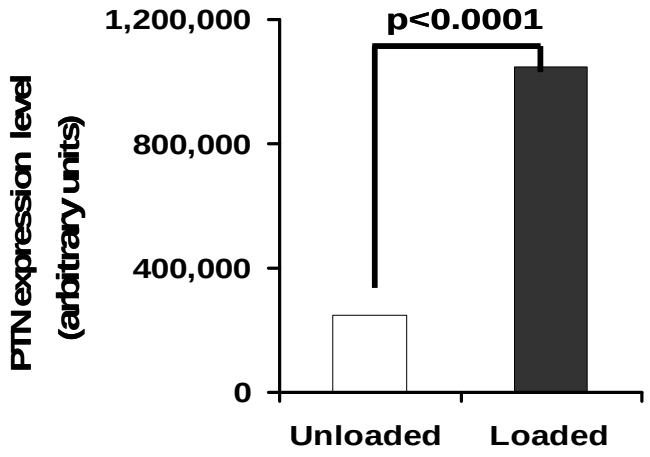

Figure 2: Mechanical loading increase pleiotrophin (PTN) expression. Whole genome microarray analysis was performed using RNA extracted from loaded and unloaded bones as described (15). Values are mean of 5 replicates per group.

syndecan and receptor protein tyrosine phosphatase ζ, are known to be expressed in osteoblasts and are known to regulate skeletal development. Furthermore, the down stream molecules of syndecan and receptor protein tyrosine phosphatase, namely PI3K and beta catenin are known to exert significant biological effects on osteoblasts.

If PTN is, indeed, responsible for recruitment of osteoblasts to specific sites of bone formation, then one would expect PTN to play a significant role in the bone formation. Based on the current literature, we propose a model of PTN action in mediating mechanical loading induced bone formation as shown in Figure 1. To date, bone formation response induced by mechanical loading is well-established [3]. The molecular components that are responsible for increasing bone's in vivo adaptive response to loading, however, are not well understood. Recently, using microarray, we have shown that the PTN gene was increased 4-fold in C57BL/6J mice in response to mechanical loading (**Figure 2**). [15]. Based on the above findings and our data that PTN expression is increased in response to mechanical loading, we hypothesize that PTN play a role in mediating anabolic effects of mechanical loading on bone formation.

To confirm the microarray data we evaluated the expression levels of PTN by real time RT-PCR in 2 different experiments as described below. In the past, we have reported that bone formation response to mechanical loading increases with time course of loading as evidenced from the expression levels of bone formation marker genes [1]. If PTN gene is associated with bone formation response, one would anticipate PTN

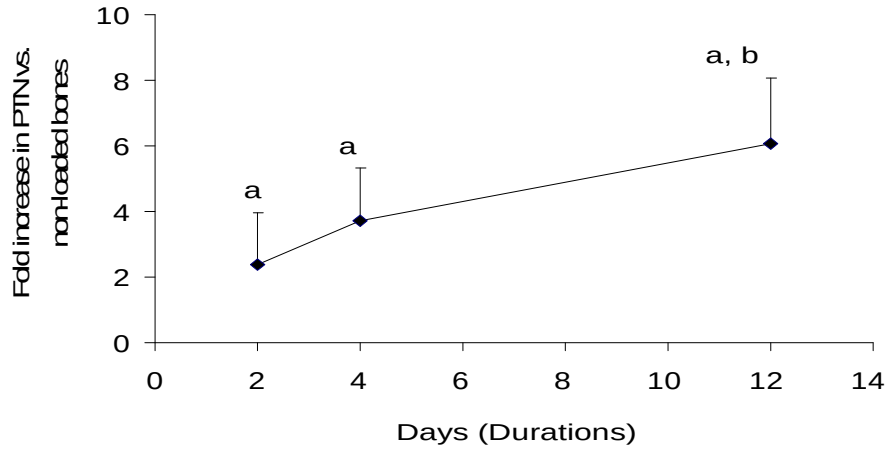

Figure 3: Expression levels of PTN gene as a function of duration of loading. The y-axis represents fold increase in the PTN gene in response to four-point bending on tibia and the x-axis represents duration of loading on 10-week female C57BL/6J mice. Values are mean ± SD, $^{a}p<0.05$ vs. non-loaded bones, $^{a}p<0.01$ vs. 2- and 4-days (ONE WAY ANOVA), N=5.

expression to increase with increase in duration of loading. We performed four-point bending as a time course (2, 4-, and 12-days) on 10-week female C57BL/6J mice. Expressions levels of PTN were measured by real time PCR using gene specific primers. The expression levels were calculated as fold change by comparing the difference

between loaded and non-loaded tibiae. Furthermore, the expression data are normalized with house keeping gene (b-actin and PPIA) to assure that the effect is not due to change in RNA quality or quantity. The results from this study show PTN gene expression was in fact increased by 2-, 3- and 6-fold at 2-, 4- and 12-days of loading (**Figure 3**). Our results support the finding that PTN is associated with early response to mechanical loading, presumably with recruitment of osteoblast. In addition to duration of loading**,** We also performed 12 days four-point bending as a function of age (10-, 16- and 36 wk) in female C57BL/6J mice to determine their expression levels. The results from our study show PTN gene expression was in fact increased by 6-, 7- and 10-fold in 10-, 16- and 36-wk female C57BL/6J mice, respectively. Mechanical loading induced PTN expression was increased in all three age groups of mice (**Figure-4**).

Overall, these data demonstrate that PTN is a mechanoresponsive gene in the bones of mice. In contrast to this in vivo finding, an in vitro study using cultured human osteoblast cells have shown that PTN expression decreases in response to mechanical stimulation [25]. Although we cannot fully explain this discrepancy between our data and the in vitro study, possible explanation includes: 1) osteoblast responsiveness to mechanical loading may differ in vivo vs. in vitro. 2) Type of mechanical loading and the amount of strain utilize were different between the two studies.

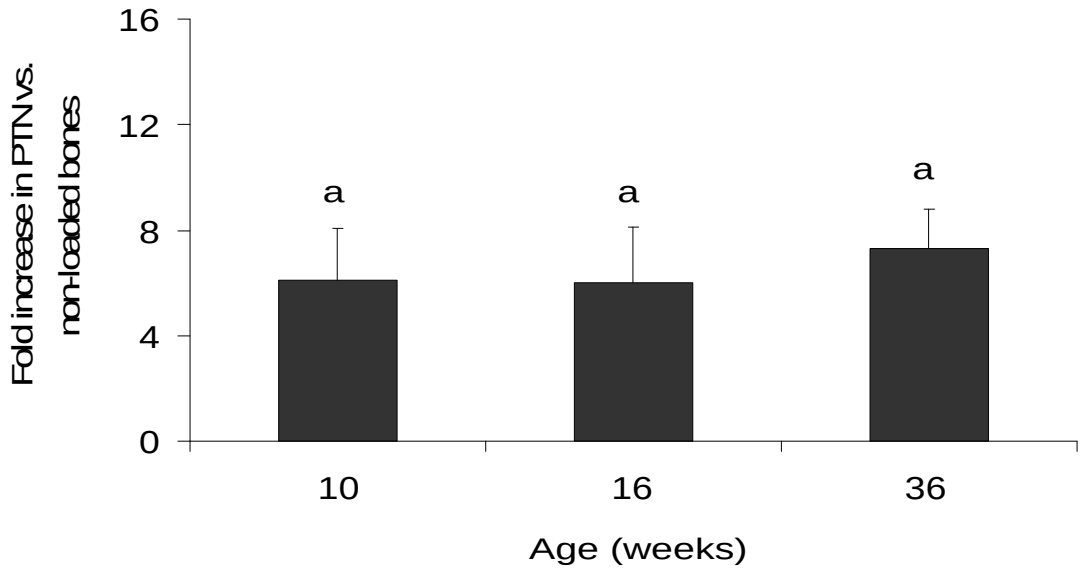

Figure 4: Expression levels of PTN gene as a function of age.
The y-axis represents fold increase in the PTN gene expression in response to 12 days of four-point bending on tibia and the x-axis represents varying age groups of female C57BL/6J mice. Values are mean ± SD. $^{a}p<0.05$ vs. non-loaded bones, N=5.

In the past, we have reported that four-point bending caused a 15% increase in bone mineral density (BMD) in B6 female mice. This increase in BMD in the C56BL/6J mice is the result of an increased periosteal circumference (PC), which in turn leads to an

increased cortical thickness (CTh). In the C3H/HeJ mice, in contrast to the B6 mice, there was no observed change in the BMD. However, there were changes in the PC and CTh although the magnitude of these changes was much less than those of C57BL/6J mice. Both B6 and C3H/HeJ mice showed a similar changes in resorption at the endosteal circumference, while the magnitude of periosteal response in the C3H/HeJ mice was much less, thus explaining the cause for no change in BMD in C3H/HeJ mice. This, then, raises a question whether the greater magnitude of bone response to loading in C57BL/6J mice may be related to the expression levels of PTN. We therefore, using real time PCR, evaluated the expression levels PTN using a good responder (B6) and a poor responder (C3H) mouse strains. We found increases in PTN expression in response to four-point bending from 12 days loading. We found that the expression of PTN gene was greater in C57BL/6J (6-fold) mice than C3H/HeJ mice (2.95 fold). The fact that PTN was expressed to a greater extent in the good responder (B6) than in the poor responder (C3H) provided additional strong evidence that PTN is associated with the skeletal anabolic response to mechanical loading. Together, this finding and data from the above experiments though show that PTN mediate skeletal anabolic response to loading, direct evidence is lacking.

To test the hypothesis that PTN is a mediator of skeletal anabolic response, we used targeted PTN knock out mice to evaluate PTN role in mechanical loading. PTN gene knock out (KO) mice (PTN-129 in B6 background) were generated by Dr. Thomas F. Vogt and the breeding pairs were kindly provided by Princeton University, NJ, USA, for our studies. PTN KO mice were crossed with wild type C57BL/6J mice (Jackson laboratory, Bar Harbor, ME) to generate the heterozygotes. These were crossed with each other to generate 25% homozygous PTN KO mice, 50% heterozygous and 25% littermate wild type mice. The body weights of PTN KO and control mice used for this study were 18.18 ± 0.97 g and 19.0 ± 1.39 g, respectively. The body weights of PTN KO and control mice were not statically different. All mice were housed under the standard conditions of 14-hour light and 10-hour darkness, and had free access to food and water. The experimental protocols were in compliance with animal welfare regulations and approved by local IACUC.

At 3-weeks (wks) of age, DNA was extracted from tail of female mice, using a PUREGENE DNA purification kit (Gentra System, Inc., Minneapolis, MN) according to the manufacturer's protocol. Polymerase chain reaction (PCR) was performed to identify PTN KO mice from wild type or heterozygous mice. Primers specific for neomycin gene (forward 5' CTT GCT CCT GCC GAG AAA GTA T 3' and reverse 5' AGC AAT ATC ACG GGT AGC CAA C 3' with a PCR product of 369 bp). Primers specific for PTN gene (forward 5' TCT GAC TGT GGQA GAA TGG CAG T 3' and reverse 5' CTT CTT CCA GTT GCA AGG GAT C 3' with a PCR product of 147 bp) were used for genotyping. The following conditions were used to perform the PCR reaction: 95°C for 2 minutes; 35 cycles at 95°C for 40 sec, 57°C for 40 sec, 72°C for 40 sec; 70°C for 40 sec. The PCR products were run on a 1.5% agarose gel and the image taken with a ChemiImager 4400 (Alpha Innotech Corp., San Leandro, CA).

It is well established that the amount of mechanical strain exerted by a given load is largely dependent on the cross sectional area (moment of inertia) such that a mouse with a large cross sectional area will experience lower mechanical strain and vice versa for small circumference. In order to assure that the difference in the bone responsiveness to loading between PTN KO mice and controls is not due to difference in the mechanical strain, we measured the bone size by pQCT at tibia mid diaphysis and calculated the mechanical strain using a mathematical model (Stephen C. Cowin: Bone Mechanics Hand book, 2nd edition, 2001, chapter: Techniques from mechanics and imaging) for both sets of mice before the loading. We found that there was no significant difference in the bone size (4.55 mm vs. 4.69 mm, p=0.50) as well as in the mechanical strain for 9N (6310με vs.6351 με, p=0.91) between the PTN KO mice and controls. Therefore, we chose same mechanical load to induce bone formation in both sets of mice.

To determine if mechanical loading induced increase in PTN expression contributes to anabolic effects of mechanical loading, we performed four-point bending using a load (9N) at 2 Hz frequency for 36 cycles, once a day, for 12 days under inhalable anesthesia (5% Isoflurane and 95% oxygen). The right tibia was used for loading and the left tibia as internal non-loaded control. If PTN is an important mediator of skeletal

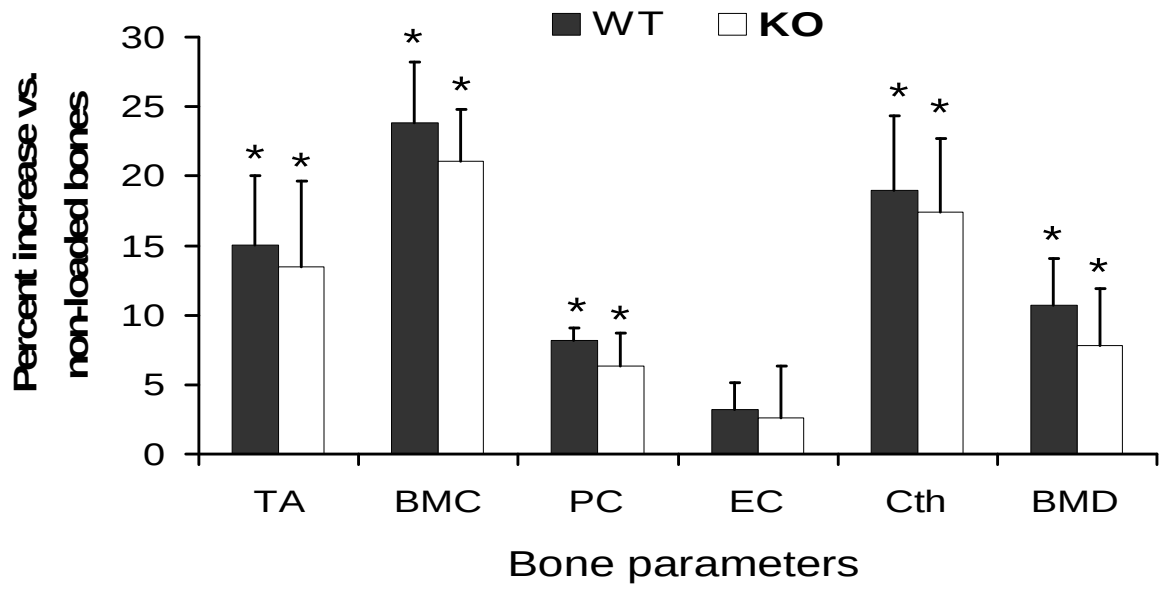

Figure 5: Changes in bone parameters in response to ML on 10-wk female PTN KO and control mice. Values are mean ± SD. The y-axis represents percent increase in bone parameters in response to four-point bending on tibia and x-axis represent skeletal parameters. TA, Total area; BMC, bone mineral content; PC, periosteal circumference; EC, endosteal circumference; Cth, cortical thickness and BMD, bone mineral density. *p<0.05 vs. corresponding non-externally loaded tibiae, N=7.

anabolic response to loading, we anticipated PTN KO mice to show reduced anabolic effects of mechanical loading on bone. We found that there was, indeed, a small reduction in mechanical loaded response, measured by pQCT, in the PTN KO mice (**Figure 5, table-1**); however, these changes were not statistically significant.

Table 1: Changes in bone parameters in response to loading between PTN KO and control mice

Bone parameters		PTN KO		WT	
		Mean ± SD		Mean ± SD	
		Non-Loaded	Loaded	Non-Loaded	Loaded
Bone mineral content	(mg/mm^3)	1.16 ± 0.10	1.40 ± 0.07*	1.17 ± 0.14	1.44 ± 0.13*
Periosteal circumference	(mm)	4.55 ± 0.23	4.83 ± 0.23*	4.69 ± 0.21	5.03 ± 0.26*
Endosteal circumference	(mm)	3.36 ± 0.18	3.44 ± 0.20	3.53 ± 0.17	3.69 ± 0.24
Total vBMD	(mg/cm^3)	881 ± 52	949 ± 55*	839 ± 40	926 ± 27*
Cortical thickness	(mm)	0.27 ± 0.01	0.31 ± 0.01*	0.25 ± 0.01	0.29 ± 0.01*

*$p<0.05$ vs. corresponding non-externally loaded tibiae, N=7

We further extended our study to determine whether PTN has any influence on the cancellous or woven bone formation induced by mechanical loading between the two sets of mice. To date, several conventional methods, though routinely used, none can analyze the architecture of the newly formed bone in response to loading, to the extent that Micro-CT, a high resolution tomography image system (Scanco Invivo CT40, Switzerland). Routine calibration was performed once a week using a three-point calibration phantom corresponding to the density from air to cortical bone. Bones (PTN KO and controls) were immersed in 1X PBS to prevent them from drying and scanning was performed using 75Kv X-ray. A scout view was performed and 400 slices with a slice increment of 10.5μm were taken starting 3 mm away from the tibia-fibular junction and progressing towards proximal. After acquiring the radiographic data, images were reconstructed by using 2-D image software (as described by manufacturers). The areas of the loaded region of bone were outlined within the midshaft compartment. Every 10 sections were outlined, and the intermediate sections were interpolated with the contouring algorithm to create a volume of interest, followed by three dimensional analyses using Scanco in vivo software. Parameters such as bone volume/total volume (BV/TV %), trabecular number (Tb.N, mm^{-1}), trabecular thickness (Tb.Th, μm), trabecular space (Tb.Sp, μm), were evaluated in the loaded and unloaded bones of both sets of mice. In **figure 6,** we show the newly formed bone on the tibia lateral side of the loaded bones of both PTN KO and control mice. However, we did not observe any difference in the amount of newly formed bone in response to loading as measured from the above parameters. This finding suggests that the microarchitecture of newly formed bone is not influenced by the lack of PTN.

A potential explanation for the lack of significant differences between the control and KO mice is that PTN disruption could lead to increased expression of other molecules which share similar functional properties to compensate for the loss of PTN. For example, midkine belong to the family of HB-GAM as PTN that has been shown to have similar functional properties. Accordingly, we evaluated the expression levels of

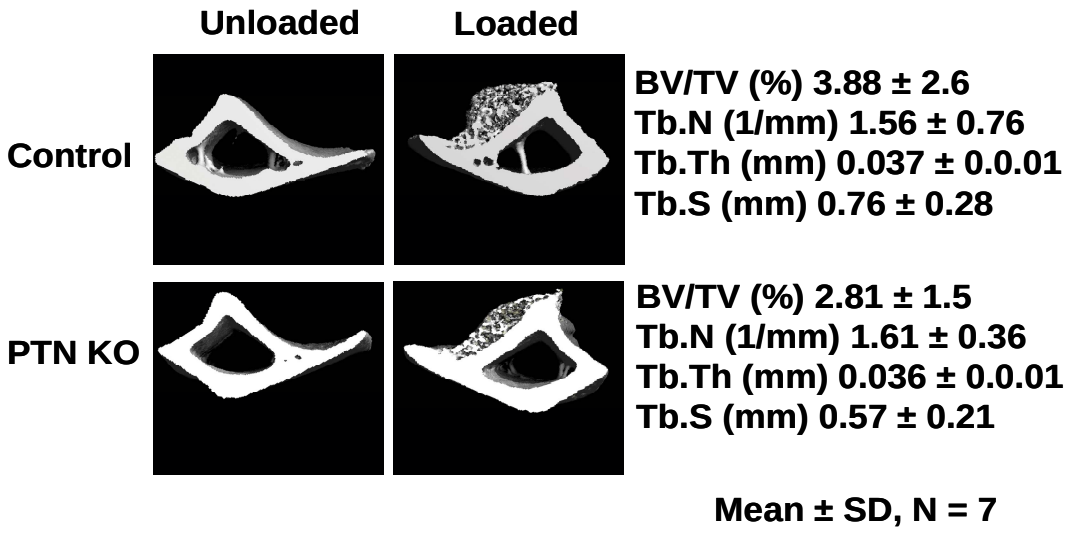

Figure 6: Micro-CT analysis of skeletal response to loading between PTN KO and control mice. Values are mean of 7 replicates per group. BV/TV, bone volume/total volume; Tb. N, trabecular number; Tb.Th, trabecular thickness and Tb.S, trabecular space

midkine between PTN KO and control mice. The result of this study showed significantly increase in the PTN KO mice when compared control mice in response to loading (**Figure 7**). Similar to our findings, other studies have shown that mice with midkine or PTN deficiency have normal low auditory response while mice with both gene deficits showed impaired auditory response [26]. A similar observation has been also reported with regard to fertility. Mice with disruption of both genes were infertile while mice with deficiency in either midkine or PTN gene were able to produce similar number of offspring's [27]. Another study has shown that mouse with absence of PTN gene resulted in normal skeletal growth and this is likely due to an increase in midkine expression as evident from their microarray data [28]. Overall, these findings suggest that factors of the same family are exhibiting overlapping function and thus, interfering with the activity of one factor may not necessarily lead to disruption of physiological activities such as bone formation response to loading. The issue of whether disruption of both PTN and midkine will exert a significant deficit in the skeletal anabolic response to mechanical loading compared to individual knock out requires further study.

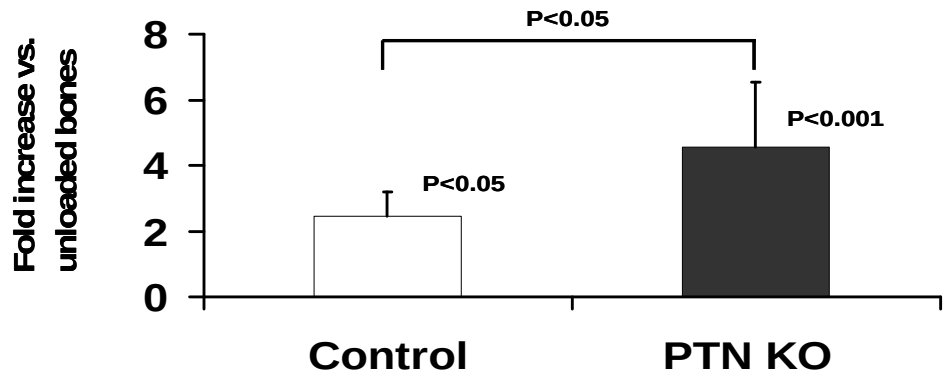

Figure 7: Increase in midkine expression in response to mechanical loading. RNA from unloaded and loaded bones of PTN KO and control mice were subjected to real time RT-PCR using midkine specific primers. Values are fold changes compared to corresponding unloaded bones, N=6.

2. Ras association domain family 1 isoform C (RASSF1C) and skeletal anabolic response to loading

In the background, we have mentioned that using the QTL approach, we have identified several chromosomal regions that regulate bone adaptive response to loading in the B6XC3H intercross [13, 14]. Among these QTL regions, we have identified several receptor coupled G-protein as potential candidate genes. Previous studies have shown that activation of G-protein coupled receptor leads to the stimulation of Ras signaling, which then activates multiple downstream signaling pathways. These pathways, MAPK, PI3K and RAF, regulate diverse cellular function and, according to recent studies, also regulate the bone adaptive response to mechanical loading [29-32] [15]. Studies have also shown that these downstream signaling pathways, mediated by Ras, require the binding of a cytoplasmic protein called Ras associated protein (RASS) for activation. Several of these proteins have been identified. Among these, our group has shown that RASSF1C is expressed in many types of osteoblast cells and it regulates osteoblast cell proliferation [33]. Bone formation induced by mechanical loading results from an increase in osteoblast number which has been shown to involve the Ras-Raf-MAPK signaling pathways. We, therefore, predicted that RASSF1C could be involved in the molecular pathway for bone anabolic response to loading.

To test this prediction, we generated a trangenic mice, in which RASSF1C expression is driven by type-I collagen promoter. To **generate this trangenic mouse lines** to over express RASSF1C specifically in osteoblast cells, we amplified the complete coding sequences of RASSF1C from our cDNA clone by PCR, tagged with a Flag synthetic epitope at tits amino terminus, and clonsed the Flag/RASSF1C fusion gene into the place of LacZ in pwhere expression vector (Invitrogen, San Diego, CA). We then inserted a heterzoygous promoter containing CMV enhancer and the actin/globin intron.

The entire transgene (9.3kb)in pwhere/2.3 Col1A1/Flag/RASSF1C also contains two mH19 insulators derived from mouse Igf2 gene on either side of the Flag/RASSF1C transcription unit (**Figure 8**). Both insulators are expected to protect the integrated transcriptional Flag/RASSF1C unit from negative as well as postive influences from neighboring sequences. We expect that the transgene would be expressed in a position-independent manner in vivo.

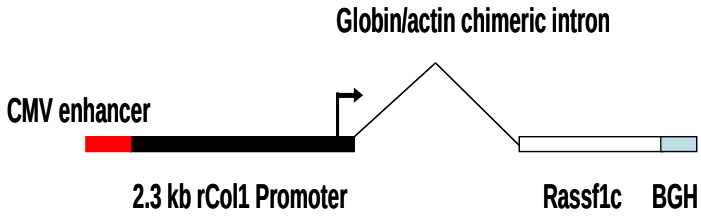

Figure 8: Transgenic construct used for over expression of RASSF1C. CMV, Cytomegalovirus; rCol1, Rat collagen type I; BGH, Bovine growth hormone and pA, poly .adenylation.

To test the transgene expression in vitro, we transfected the pwhere/2.3Col1A1/Flag/RASSF1C plasmid into pre-osteoblast cells (MC3T3-E1) and African green monkey kidney cells (Vero 2-2) and measured the expression levels by western immunoblotting with antibody against Flag (Sigma). We found that the transgene was expressed as a 32-33 kDa protein by Western immunoblot analysis in both MC3T2-E1 and Vero 2-2 cells. After confirming the transgene expression in osteoblast cells, we sent out the DNA to the transgenic Core Facility at the University of Southern California (USC) for microinjection. DNA was microinjected into the pronuclei of fertilized zygotes from C57BL X CBA/CA mice. F1 generation mice were produced by breeding the transgenic founders with C57BL/6J mice. F2 and subsequent generations of transgenic mice were generated by breeding. All mice were housed under the standard conditions of 14-hour light and 10-hour darkness, and had free access to food and water. The experimental protocols were in compliance with animal welfare regulations and approved by local IACUC. At 3-weeks of age mice were genotyped to differentiate transgenic from wild type. The genotyping protocol are descriebd earlier. Since the amount of strain produced by same load on bone is related to bone size, for example, mice with small circumfernece tend to received higher mechanical strains and vice versa in the large cirucmfernece, we measured periosteal circumference (bone size) in both sets of mice. This is to assure that the difference in the mechanical strain due variation in bone size is not be responsible for the differenec in the skeletal anabolic response to laoding between mice. But, we found no differneces in the bone size between both groups and therefore, we applied same mechanical load on both set of mice. A 9 N load was applied on the right tibia at 2Hz for 36 cycles, oncer per day for 12 days. The left tibia was used as internal control. After 48 hrs of last loading *in vivo* pQCT was performed and tibias were collected and frozen at -80°C for further study. The result from our study indicate that loading has caused a significnat increase in the bone parameters in both control and RASSF1C mice. We also found that the changes in bone parameters induced by mechanical loading were higher ($p<0.05$) in the RASSF1C trangenic mice when compared to control mice (**Figure 9**). These findings suggest involvement of Ras-Raf-MAPK signaling pathway in mechanical loading induced bone formation.

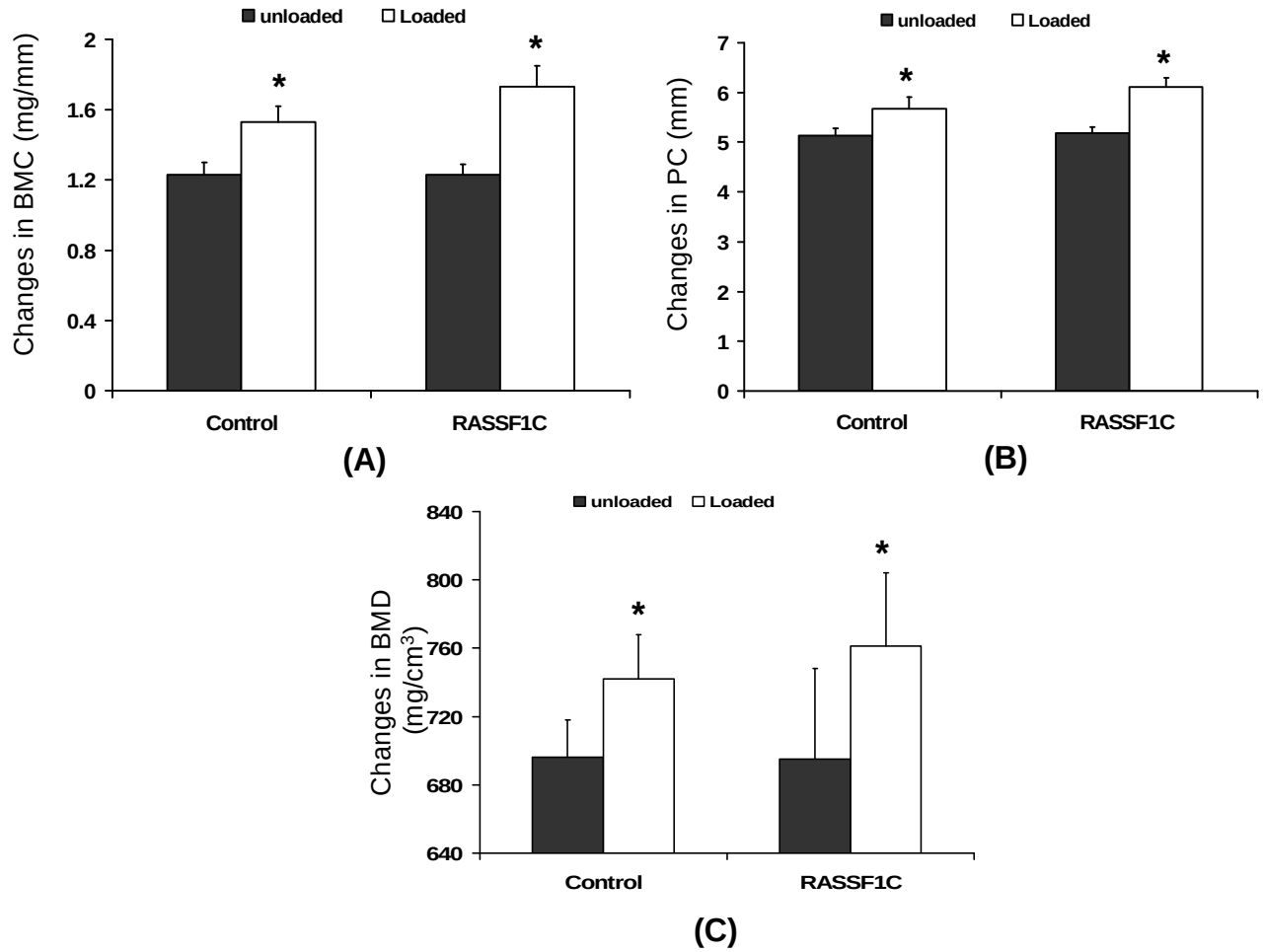

Figure 9: Changes in (A) BMC, (B) PC and (C) BMD in response to 12 days four-point bending between control and Rass1FC mice. The y-axis represents absolute change in bone parameters and x-axis represent mice group, N=5, $p<0.05$ vs. non-loaded bones.

3. Skeletal Anabolic response after cessation of loading

It is now well established that mechanical loading is an effective stimulator of bone formation. Thus, physical exercise has been used as a strategy to maintain BMD and prevent osteoporosis and fractures in men and women. Recent clinical studies in young and postmenopausal women who were subjected to treadmill exercise have shown that exercise induced benefits, such as increases in BMD and bone mineral content (BMC), are eventually lost if exercise is ceased completely [8]. Similar data was presented by Vuori et al. when reporting that unilateral leg presses done four times a week for 12 months increased bone mass (BMD) but returned to pre-training levels with only 3 months of retirement from exercise [34] [35]. Data from various independent studies in humans suggest that exercise induced bone mass benefits seem to be eroded by time. Animal studies using rat model have shown that increased femoral BMD, gained through treadmill exercise, resulted in decreased bone formation rate after deconditioning [36] [37]. We, and others, have previously shown that C57BL/6J (B6), a low bone density mouse, responds well to mechanical loading. We have reported that mechanical loading

by four-point bending causes a 10-15% increase in the tibia BMD in 10-week female B6 mice with compared to C3H/HeJ mice, after 2 weeks of loading [1]. We have extended this study, further, to examine how long this newly formed bone induced by four-point bending is maintained and their fate, after cessation of loading using the good responder, B6 mouse.

To investigate this, female B6 were purchased from the Jackson Laboratory (Bar Harbor, Me). All mice were housed under the standard conditions of 14-hour light and 10-hour darkness, and had free access to food and water. The experimental protocols were in compliance with animal welfare regulations and approved by local IACUC.

At 10 weeks of age the mice were subjected to mechanical loading using the four-point bending device as described previously [1]. The loading protocol consists of a 9.0 ± 0.2 Newton (N) force at a frequency of 2 Hz for 36 cycles performed daily under inhaleable anesthesia (5% Halothane and 95% oxygen). The loading procedure was repeated for 6 days/week with 1 day of rest for 2 weeks. Mice were euthanized 48 hours after the last loading and tissue samples were collected and stored at -20°C for further study.

After 12 days of loading, we used pQCT (Stratec XCT 960M, Norland Medical

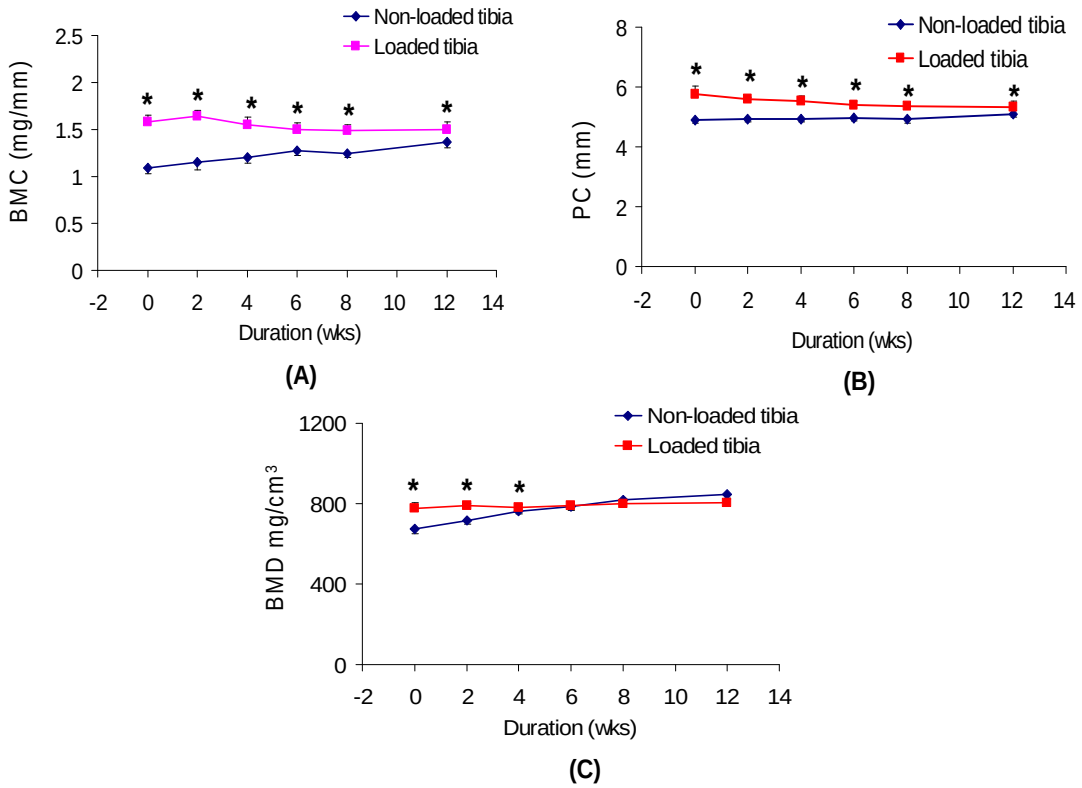

Figure 10: Changes in the bone parameters measured by *in vivo* pQCT at different time points after 12 days of four-point bending on 10 week female B6 mice. (A) Bone mineral content (BMC), (B) Periosteal circumference (PC) and (C) Bone mineral density (BMD). Values are mean ± SD, $^{*}p<0.05$ vs. non-loaded tibiae, N=5

System, Ft. Atkinson, WI) to measure loading-induced changes in the bone parameters in loaded and non-loaded tibiae, as described previously [1]. *In-vivo* pQCT measurements were performed at immediately, 2, 4, 6, 8, and 12 weeks after the last loading regimen. Data are presented as Mean ± Standard error (SE). Regression analysis, and standard t-test were used to compare differences from loading between the time points using the percentage obtained from loaded vs. non-loaded bones. We used STATISTICA software (StatSoft, Inc version 7.1, 2005) for our analysis and the results were considered significantly different at $p<0.05$.

Two weeks of four-point bending on the right tibiae when compared to left tibiae resulted in a significant increase in bone parameters, such as BMC, periosteal circumference (PC) and BMD, as shown in **Figure 10**. The mechanism through which bending induces an increase in BMD is by increasing PC and cortical thickness (Cth). Our present findings are consistent with our previous study [1]. However, we also found, that this increase in bone size and BMD did not continue over time after termination of loading. This is because BMD increased with age in non-loaded but not in the loaded tibia and as such the difference in BMD between loaded and non-loaded tibia was lost 4-wks after cessation of loading (**Figure 10**). This suggests that bone anabolic response does not continue once loading is terminated.

In this study, we found that the loading induce a 15% and 40% increase in BMD and bone size (total area), respectively, immediately after 2 weeks four-point bending (**Figure 11**). However, cessation of loading resulted in a continuous loss of both BMD and bone size (Total area). We found that the loading induced changes in volumetric BMD returned to normal at 12 weeks with a half life of 6-weeks. The change in total area (bone size) declined with a half life of 8.5 weeks and was still significantly elevated at 12 weeks. Thus, the decline in elevated TA proceeded at a much slower pace than the loading induced increase in vBMD.

In our pQCT analysis of bone parameters, using the lower threshold (180-730) we found that the magnitude of increase in PC was 18% ± 7.0 after the last loading and was very significant when compared with 7.2% ± 1.7 using the higher threshold (730-730). This is due to an increase in the low density bone mineralized at the periosteal surface. This is most prevalent at 0-time point, as seen by the red color using the pQCT threshold indicator (**Figure 12**). Consequently, we found a significant increase in bone size at 0-time point, accompanied by an increase in BMD. Over time, we found that PC increased with age in the non-loaded but not loaded tibia. As a result, the magnitude of difference between loaded and non-loaded tibia decreased with time after cessation of loading and subsequently we found no difference in the periosteal circumference of loaded tibiae between lower (PC, 9.13% ± 3.9) and higher threshold (PC, 9.8 ± 1.2). These data suggest that the newly formed bone undergoes remodeling at the periosteal site over time which leads to a reduction in bone size in order to accommodate the loading induced changes in bone shape. During this remodeling process, we found that the rate of loss in gained bone size (total area) after cessation of loading was 10% every 2 weeks for 8 weeks. These data, together, suggest that the positive effects of mechanical loading on bone size were maintained for several weeks after cessation of loading.

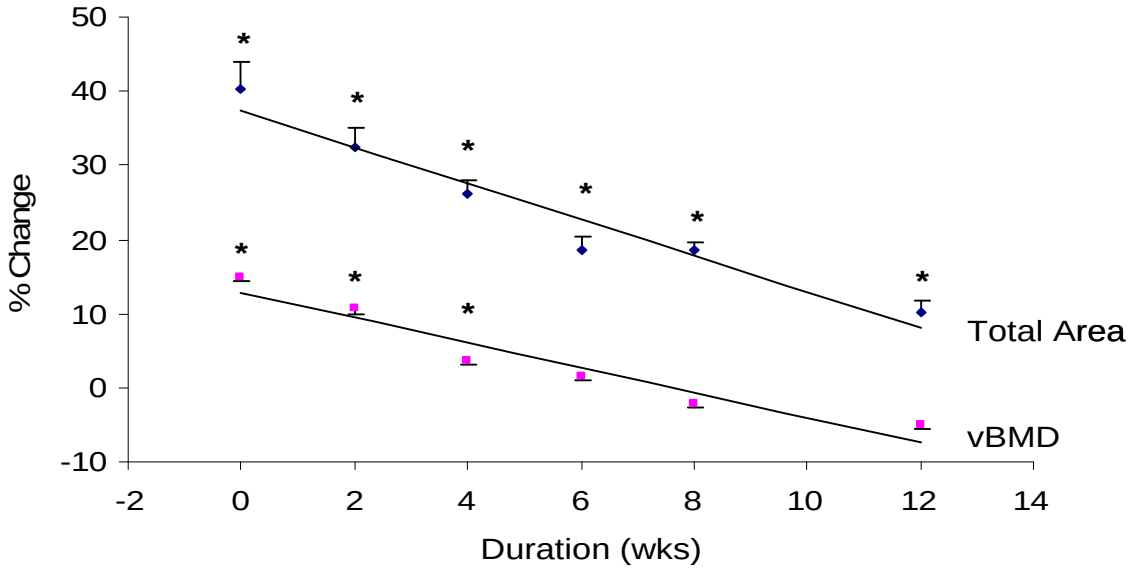

Figure 11: Percent changes in the total Area and BMD after termination of loading at different time points in 10 week female B6 mice. The y-axis represents percent change and the x-axis corresponds to duration (weeks). TA, Total area and vBMD, total volumetric bone mineral density, values are given as mean ± SE, *p<0.05 vs. non-loaded tibiae, N=5.

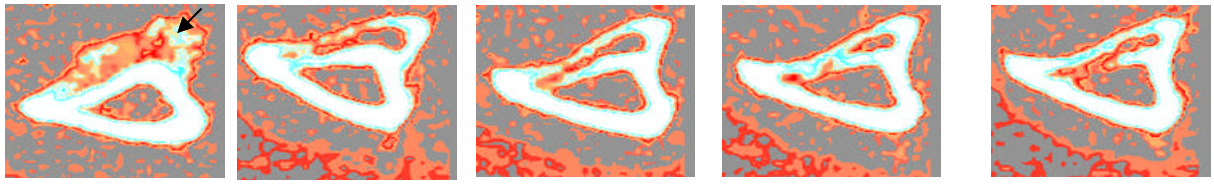

Figure 12: Cross sectional area of the loaded tibia. This figure shows the newly formed bone at the periosteal site in response to 2-weeks loading and their fate over time after cessation of loading. A, 0- week; B, 2-weeks; C, 4-weeks; D, 6-weeks and E, 8-weeks. The arrow corresponds to newly formed bone and the white color represents cortical bone.

In this study, we observed that the accommodation of this newly formed bone, gained through loading over time altered the shape of bone compared with non-loaded tibiae as seen from our pQCT cross-sectional slice. Since studies have shown that changes in bone size and BMD parameters leads to an increase in bone mechanical properties, one would expect a difference in these properties of bone immediately and 12 weeks, after termination of loading. To test this difference we used three-point bending device (Model 8840; Instron, Canton, MA, USA). The frozen tibiae stored at 4°C were thawed. The tibiae were placed on two immovable supports which is 12 mm apart. An initial 1.0N was applied on the tibiae to prevent the rotation of the bone and were

centrally loaded at a constant rate (10mm/minute) until fracture. Load displacement curves were used to calculate maximum load phenotype. The result from our study show a significant increase in the bone strength immediately after 2- weeks of loading in the loaded bones compared to non-loaded bones, as evident from maximum load data (-19.14 N ± 1.57 vs. -24.74N ± 1.14, $p<0.001$, n=4). However, this increase in bone strength was lost at 12 weeks. We found that there was no difference in the maximum load phenotype between the loaded and non-loaded bones (-22.26 ± 2.28 vs. 21.07 ± 0.70, p=0.43, n=4) at 12 weeks after cessation of loading (**Figure 13**). This is consistent with the changes in bone parameters. Together, the findings of our study indicate that the external loading-

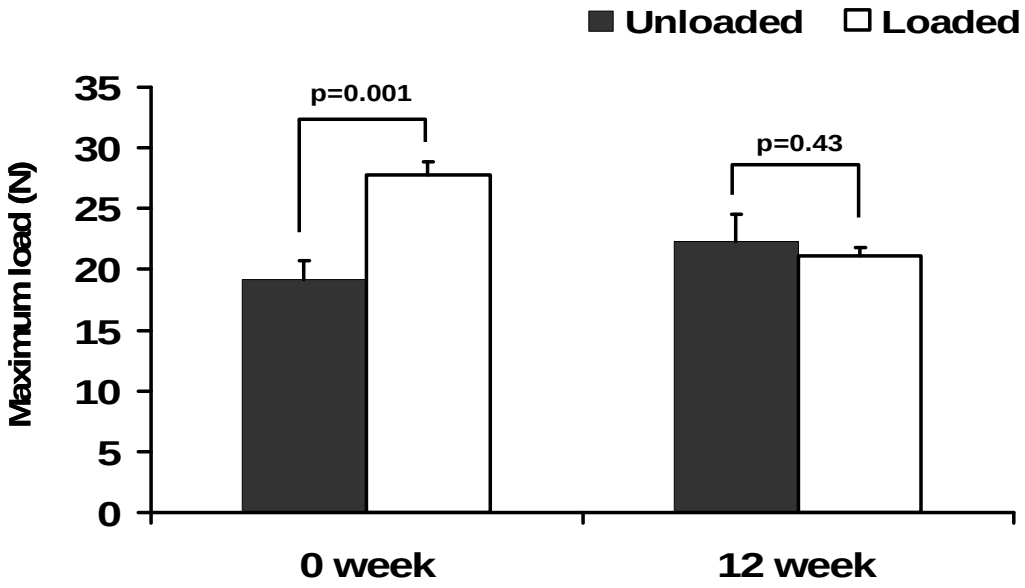

Figure 13: Changes in maximum load (N) in loaded and unloaded bone immediately and at 12 weeks after cessation of loading. Mouse tibias (loaded and unloaded tibias) were collected immediately after 2-weeks and at 12 weeks after the cessation of loading and subject to three-point testing to measure the strength of the bone. Values are mean of 4 replicate per group.

induced increases in bone are lost over a period of time but at a much slower pace than the induction of the increases; 2) While a single burst of external loading provides increased bone mass temporarily, periodic loading may be necessary to maintain long term bone strength**.**

4. Generation of IGF-I conditional Knock out mice

It is now well known from several studies that IGF-I produced by osteoblast cells is an important regulator of both prenatal and postnatal skeletal growth [38-40]. Since local IGF-I is an important regulator of bone formation and mechanical loading is a key regulator of bone formation, we and others predict that local IGF-I is essential for bone formation response to loading. To date there is abundant evidence that demonstrate IGF-I as a potential mediator of the anabolic effects of mechanical loading on bone formation. These include: 1) Rapid induction of IGF-I mRNA levels have been shown to occur in response to mechanical stimulation in bone cells *in vitro* and bones *in vivo* [31, 41, 42].

2) IGF-I treatment induced bone formation in GH-deficient normally loaded rats but not in unloaded rats, thus suggesting that skeletal unloading induces resistance to IGF-I [43, 44]. 3) Transgenic mice with elevated IGF-I expression in osteoblast exhibit increased bone formation when compared to control mice [45]. In addition to these studies, our genome-wide microarray data also revealed that IGF-I expression was increased by 2-fold ($p<0.003$) after the mechanical loading in a good responder C57BL/6J mouse. Furthermore, we also found increase in the expression levels of several other genes that are directly or indirectly, associated and/regulated by IGF-I [15]. If IGF-I gene is associated with bone formation response induced by mechanical loading, one might expect IGF-I expression to also be increased across the various ages as we previously reported that skeletal anabolic response to loading increased across these ages. Accordingly, we performed 12 days loading using four-point bending device as a function of age (16- and 36-wk) in female C57BL/6J mice. The results from our study show IGF-I gene expression was in fact increased by 1.6 to 2.1-fold ($N=5$, $p<0.05$) in 16- and 36-week female C57BL/6J mice, respectively in the loaded bone with compared to non-loaded bones (**Figure 14**). These findings suggest that 1) anabolic effects of mechanical loading on bone formation are mediated by IGF-I and 2) IGF-I is inducible to mechanical loading. Although, these findings provide indirect evidence for the role of IGF-I in mediating the bone anabolic response to loading, *the cause and effect relationship between loading-induced increases in IGF-I expression and skeletal anabolic changes is lacking*. To test for a role for locally produced IGF-I in mediating the effects of mechanical strain, we propose to generate IGF-I conditional KO mice. Our group recently developed a loxP IGF-I mice in which exon 4 of the IGF-I gene is flanked by the loxP gene (IGF-I$^{lox/lox}$). Breeding pairs of transgenic mice in which Cre recombinase driven by the procollagen, type-I αII gene (Col1α2-Cre) is available in our

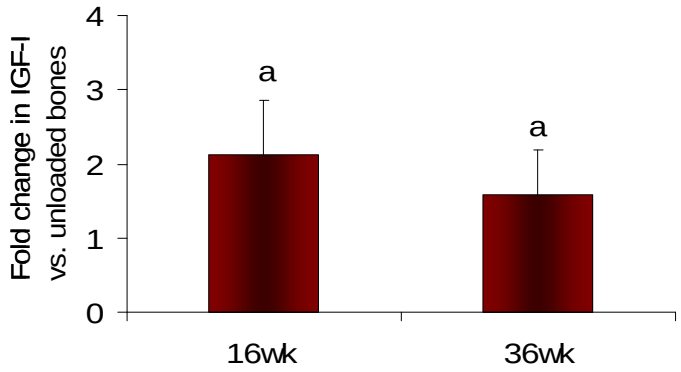

Figure 14: Increase in the mRNA levels of IGF-I gene after 12 days of four-point bending as a function of age in female B6 mice. [a]$p<0.05$ vs. unloaded bones.

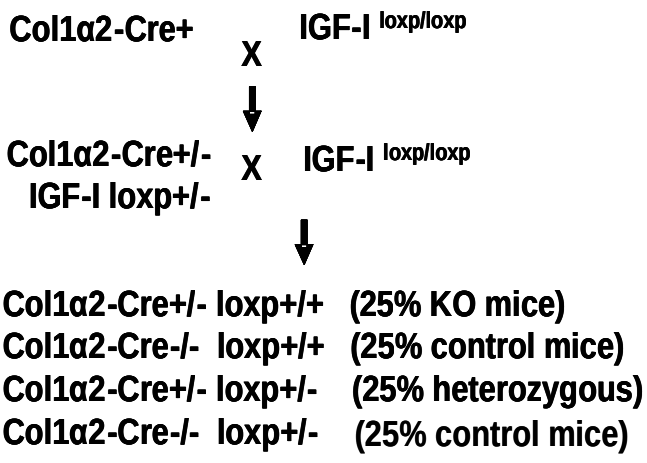

Figure 15: Generation of conditional IGF-I KO mice

laboratory. At present, we crossing this loxp IGF-I mice with Cre mice to generate conditional IGF-I KO mice and littermate controls. A schematic representation of the breeding is shown in **Figure 15**.

D. Key Research Accomplishments

1. Disruption of PTN gene alone is not sufficient to impair skeletal anabolic response to loading in mice.
2. Disruption of both PTN and midkine may be necessary to fully evaluate the role of heparin binding growth associated molecules in mediating anabolic effects of mechanical loading in bone.
3. Rass1FC, an upstream signaling molecule, is associated in mediating skeletal anabolic response to loading.
4. Our study shows that the external loading induced changes in bone are lost over a period of time and not rapidly after cessation of loading.
5. A single burst of external loading provides increased bone mass temporarily, periodic loading may be necessary to maintain long term bone strength.
6. We have established breeding colonies of IGF-I loxP and collagen 1 Cre mice to generate conditional knockout mice to evaluate the effects of locally produced IGF-I in regulating skeletal anabolic effects of mechanical loading.

E. Reported outcomes

1. Poster presentation at 29th meeting of ASBMR for the paper entitled "The Increased vBMD and Bone Area due to Mechanical Loading Gradually Decreased Following Cessation of Loading 2007.
2. Oral presentation at 30th meeting of ASBMR for the paper entitled "Anabolic Response to Skeletal Loading in Mice with Targeted Disruption of Pleiotrophin Gene, 2008.
3. Kesavan C and Subburaman Mohan. Anabolic Response to Skeletal Loading in Mice with Targeted Disruption of the Pleiotrophin gene, *BMC Research Notes*, [Accepted, 2008].
4. Kesavan C and Subburaman Mohan. Increased vBMD and Bone Area due to Mechanical Loading Gradually Decreased Following Cessation of Loading on 10 week C57BL/6J Mice. Manuscript writing is in progress.

F. Conclusions

Our studies using targeted disruption of pleiotrophin gene reveal that pleotrophin is not a major mediator of skeletal anabolic response to loading. Our studies using transgenic mice overexpressing RassF1C reveal that Ras signaling is involved in mediating skeletal anabolic effects of mechanical loading. We have also found that new bone formed in response to mechanical loading is lost after cessation of loading, thus suggesting that periodic loading is necessary to maintain the positive effects of loading on the skeleton.

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II. Molecular Genetic Studies of Bone Mechanical Strain ~ In vitro studies

A. Introduction:

This portion of the report summarizes our progress made during the past year on the *in vitro* investigation into the molecular mechanism that mediates mechanical stimulation of osteoblast proliferation and differentiation, using primary mouse osteoblasts as the cell model and fluid shear stress as a surrogate of mechanical stimulus.

B. Technical Objectives: The original specific objectives for the *in vitro* studies during the past year of this grant are as follows:

1. To optimize the *in vitro* siRNA techniques for the application to mouse osteoblasts in conjunction with our shear stress technology.
2. To select one or more ESTs (or known genes) for further study from our microarray data.
3. To apply the siRNA technique to suppress the candidate EST expression or known gene and then evaluate the functional role of this EST or known gene in osteoblast proliferation, differentiation, and apoptosis.
4. To continue to advance our protein-tyrosine phosphorylation studies in order to identify signaling proteins that show differences in protein-tyrosine phosphorylation levels in response to mechanical strain in bone cells isolated from those mouse strains which exhibit differential responses to the *in vitro* mouse strains. Changes in protein-tyrosine phosphorylation levels will be compared to in vitro parameters of osteoblast proliferation, differentiation, and apoptosis.

C. Body:

In our original experimental approach, we plan to identify potential candidate genes from our microarray data and to determine the effect of suppression of expression of the candidate gene in osteoblasts by the siRNA technology on their anabolic response to fluid shear stress. From our microarray and subsequent studies, which have been included in the previous report, we have compelling evidence that four anabolic signaling pathways, i.e., the IGF-I, estrogen receptor, Wnt, and BMP/TGFβ signaling pathways are downstream to the "mechanosensitivity" genes contributing to the good and poor bone formation response in B6 and C3H mice, respectively (1), and that the leptin receptor gene may be one of the "mechanosensitivity" modulating gene located with the chromosome 4 QTL region that contributes in part to the differential anabolic response to mechanical loading in C57BL/6J (B6) and C3H/HeJ (C3H) mice. During the previous year of our investigation, we have accumulated a large body of compelling *in vivo* and *in vitro* evidence that the leptin receptor (Lepr) signaling mechanism may function as a negative regulator of mechanotransduction. Most of our *in vitro* evidence has been summarized in the previous progress report. Therefore, this report will focus on our *in vivo* evidence supporting a negative regulatory role for the leptin-Lepr signaling. Inasmuch as our data strongly implicating an important negative regulatory function of

the Lepr signaling on mechanical stimulation of bone formation, there are two concerns about our experimental designs. First, our conclusion that the Lepr signaling is an essential regulatory mechanism of mechanical stimulation of bone formation *in vivo* was based entirely on studies with leptin-deficient (ob/ob) mice rather than Lepr-deficient mice. Second, our in vitro work used a steady fluid shear stress as the mechanical stimulus. It is now generally assumed that pulsatiled shear stress rather than steady shear stress is physiologically relevant. Therefore, it is necessary to confirm our *in vitro* studies with the pulsatiled flow. Consequently, work of past year has been largely focused on two revised objectives:

1. To generate a colony of Lepr-deficient mice for investigation.
2. To develop a pulsatiled fluid flow system for subsequent confirmation studies.

The following section summarizes our progress towards some of the original objectives as well as the revised objectives:

Specific Objective #1. To demonstrate if the leptin/Lepr signaling plays a negative mechanosensitivity modulating function in mechanical stimulation of bone formation in mice *in vivo*.

Our previous progress report has summarized our strong *in vitro* evidence that the Lepr signaling may function as a negative mechanosensitivity modulating pathway. To demonstrate that the Lepr signaling has a physiological regulatory role in mechanical stimulation of bone formation, we evaluated whether leptin-deficiency would alter bone mechanosensitivity *in vivo*. Leptin-deficient ob^-/ob^- mice rather than the *Lepr*-deficient db^-/db^- mice were used in this study, simply because of convenience, as we have already established a breeding colony of ob^-/ob^- mice in our laboratory, and we have previously characterized the bone phenotype of these ob^-/ob^- mice (2). Heterozygous male and female leptin-deficient (ob^-/ob^-) breeder mice (B6.v-Lep^{ob}/J) were obtained from the Jackson Laboratories (Bar Harbor, ME) and maintained at the J. L. Pettis Memorial V. A. Medical Center. Leptin-deficient mice were bred, and the homozygous leptin-deficient genotype was confirmed by reverse-transcriptase polymerase chain reaction (RT-PCR) as previously described (3). Our previous investigation of the bone phenotype of ob^-/ob^- mice revealed that adult male ob^-/ob^- mice lacked the sex-related differences in the greater periosteal expansion and that the loss of sex-related bone size differences in male ob^-/ob^- mice appeared to be due to a defective androgen signaling in male ob^-/ob^- mice (2). To avoid potential sex-related effects, this study used only adult (10-week-old) female leptin-deficient ob^-/ob^- mice. Age-matched female B6 mice (also from the Jackson Laboratories) were included as the background strain control for comparison.

The osteogenic response to loading (in the form of 2-week four-point bending exercise) on tibia of adult female leptin-deficient ob^-/ob^- mice was compared with that on tibia of age-matched female C57BL/6J (B6) background genetic strain control mice. However, Fig. 1A shows that the size (i.e., periosteal circumference) of tibia of ob^-/ob^- mice was significantly bigger (by ~14%) than that of age-matched female B6 tibia.

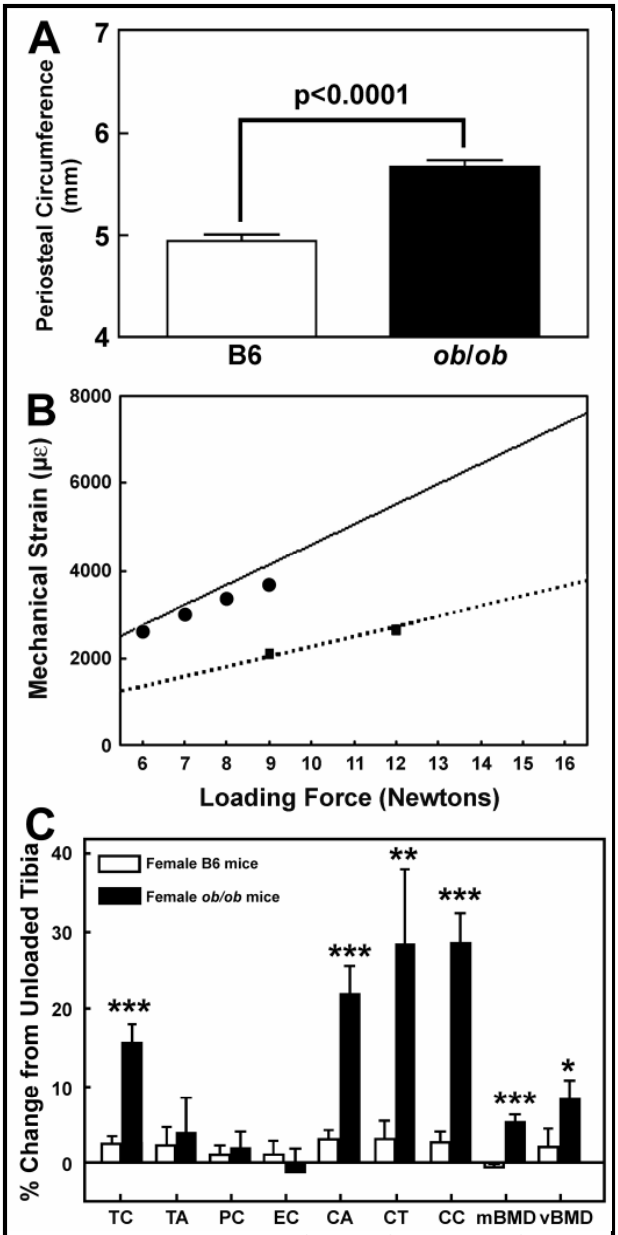

Figure 1. The periosteal circumference of 10-week-old female B6 and ob/ob mice (A), the calculated and experimental relationship between loading force (in Newtons) and mechanical strain sensed by the bone (in με) in female B6 and *ob/ob* mice (B), and the bone responses in the tibia (by pQCT) of female *ob/ob* mice (filled bars) after a 2-week 4-point bending exercise as opposed to those in the tibia of female B6 mice (open bars) (C). In B, the solid line represents the calculated strain for each loading force in female B6 mice, and the dotted line is the calculated strain for each loading force for female *ob/ob* mice. Filled circles are the actual strain sensed by the bone of female B6 mice measured with a strain gauge, whereas filled squares are the actual strain sensed by the bone of female *ob/ob* mice. In C, the bending exercise was performed in six ob/ob mice and twelve B6 mice. The results are shown as relative percent change from the unloaded tibia of individual mouse (mean±SEM). TC = total bone mineral content; TA = total area; PC = periosteal circumference; EC = endosteal circumference; CA = cortical area; CT = cortical thickness; CC = cortical content; mBMD = material bone mineral density; vBMD = volumetric BMD. *p<0.05; **p<0.01; ***p<0.001.

Because the bone formation response is determined by mechanical strain sensed by the bone rather than the loading force *per se*, we measured, with a strain gauge, the actual strain sensed by the loaded tibias of adult female ob^-/ob^- mice as opposed to age-matched female B6 tibia at various loads. Fig. 1B shows that: 1) the determined strain sensed by the bones compared well with the calculated strain at various loading forces on each mouse strain based on respective bone size, and 2) the tibia of B6 mice with a larger bone size had indeed experienced a much higher strain at any given load than tibias of ob^-/ob^- mice. Hence, the loading force for each mouse strain must be adjusted to ensure that similar mechanical strain was applied to tibia of each mouse strain. Our past studies in B6 mice used 9-N (4,5). If a 9-N load were to be used in B6 mice, a loading force of ~16-N would be needed for ob^-/ob^- mice. Loading forces of >12-N would break these bones. Thus, we used a 9-N load (~2,100 με) for female ob^-/ob^- mice and a 6-N load (~2,500 με) for female B6 mice.

Consistent with the previous dose-dependent studies which showed that the 6-N force is insufficient to produce a bone formation response in adult B6 mice (4,5), this dosage of strain had no effect on any of the pQCT parameters in adult female B6 mice (Fig. 1C). Conversely, this strain increased significantly total bone mineral content, cortical area, cortical content, cortical thickness, and material and volumetric bone mineral densities at the site of loading in ob^-/ob^- mice, indicating that ob^-/ob^- mice had an enhanced mechanosensitivity compared to B6

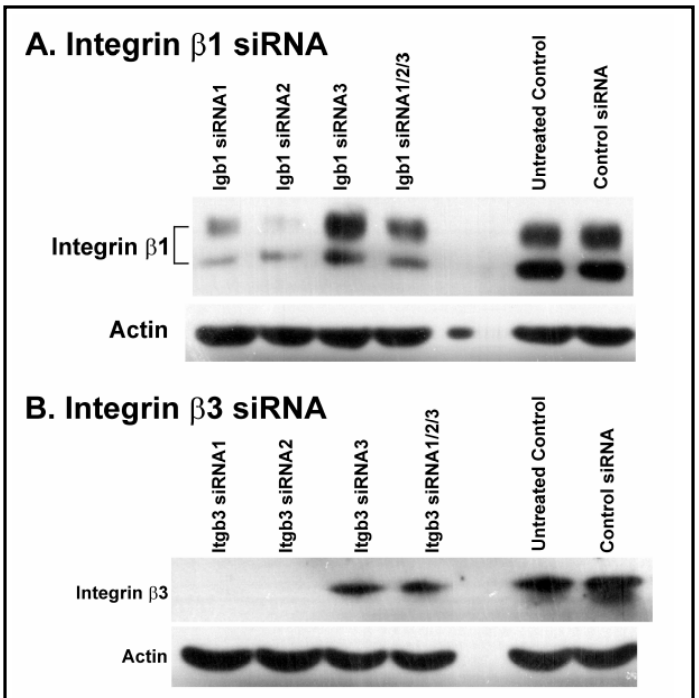

Figure 2. Suppression of integrin β1 (1) and β3 (B) expression by specific siRNAs in primary B6 osteoblasts. A set of 3 siRNAs each against mouse integrin β1 or β3 along with a control siRNA were designed and synthesized by Qiagen. B6 osteoblasts were transfected with each siRNA alone or in combination using the HiPerFect transfection reagent (Qiagen) with the total siRNA concentration in each treatment was 5 nM. After 24 hr, cellular integrin β1 or β3 and actin levels were analyzed by Western blots. As reported previously, integrin β1 existed as duplets (5).

mice.

<u>Specific Objective 2. To optimize the *in vitro* siRNA techniques for the application to mouse osteoblasts in conjunction with our shear stress technology</u>.

One of our original Technical Objectives was to apply the *in vitro* siRNA techniques for functional testing of candidate genes in mechanotransduction. Consequently, we have also initiated work to develop effective protocols for the *in vitro* siRNA techniques for the application to primary mouse osteoblasts.

Because integrin signaling has been demonstrated to be important for mechanotransduction, we sought to develop an *in vitro* siRNA protocol using integrin β1 as the target gene for testing in primary mouse osteoblasts. For this work, the siRNAs were pre-designed by Qiagen based on the proprietary HiPerformance siRNA Design Algorithm. A set of three siRNAs were obtained: *Igtb1* siRNA1 (target sequence: CTG CTA ATA AAT GTC CAA ATA), *Igtb1* siRNA2 (target sequence: CTG GTC CAT GTC TAG CGT CAA), and *Igtb1* siRNA3 (target sequence: CCA GCT AAT CAT CGA TGC CTA). Two of the test siRNAs (i.e., *Igtb1* siRNA1 and 2) were effective in suppressing integrin β1 expression. The 24-hr pretreatment with *Itgb*1 siRNA2 suppressed integrin β1 expression by >90% (Fig. 2A) and blocked the fluid shear-induced proliferation, and differentiation (Fig. 3). Therefore, these findings indicate that our *in vitro* siRNA strategy can be used to effective knock down integrin β1 gene expression in primary osteoblasts, and also provide compelling evidence that integrin β1 is essential in mechanotransduction. To further test our protocol, we also applied the same strategy to successfully knock down integrin β3 expression in primary mouse osteoblasts [with *Itgb3* siRNA1 (target sequence: CCG CTT CAA TGA AGA AGT GAA) and *Itgb3* siRNA 2 (target sequence: CAG AGG ATT GTC CTT CGA CTA), but not *Itgb3* siRNA3 (target sequence: CGC CGT GAA TTG TAC CTA CAA)] in B6 osteoblasts (Fig. 2B).

We next evaluated whether knocking down Lepr expression in B6 osteoblasts by specific siRNAs would affect the fluid shear-induced Erk1/2 phosphorylation. For this

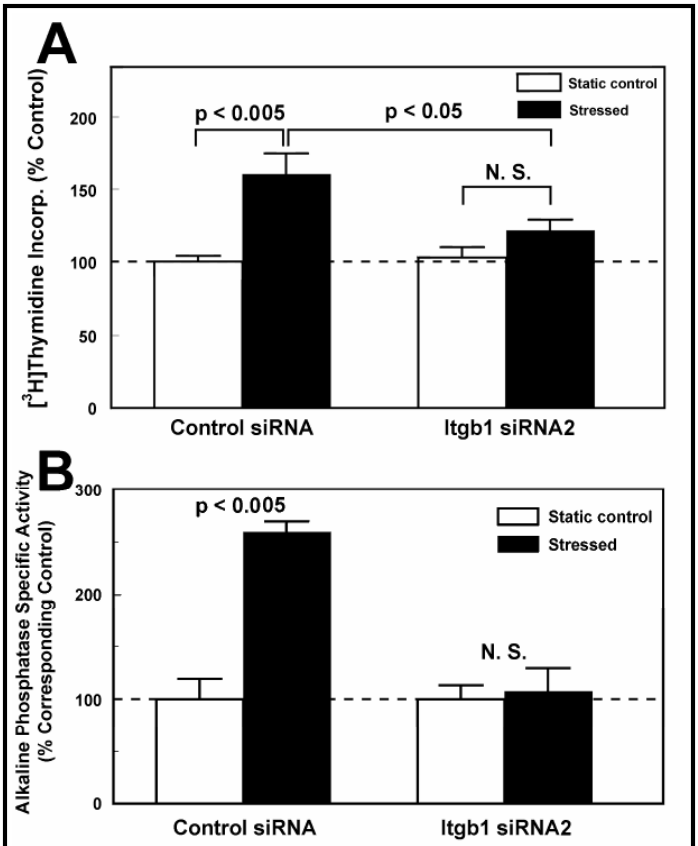

Figure 3. siRNA-mediated integrin β1 gene knockdown in B6 osteoblasts blocked fluid shear-induced prolifera-tion (A) and differentiation (B). Suppression of integrin β1 expression in B6 osteoblasts were done with 5 nM Itgb1 siRNA2 for 24 hrs. Results are shown as mean±SEM (n=7 for thymidine incorporation, and n=4 for ALP specific activity).

work, a set of three small-interfering RNA duplex (siRNA) specific for mouse *Lepr* [i.e., *Lepr* siRNA1 (target sequence: CCC GAG CAA ATT AGA AAC AAA), *Lepr* siRNA2 (target sequence: ATC GAT GTC AAT ATC AAT ATA), and *Lepr* siRNA3 (target sequence: TTG AAG CTA AAT TTA ATT CAA)] and a non-silencing control siRNA without any homology to known mammalian genes were designed and synthesized by Qiagen. For the siRNA experiment, primary B6 or C3H/HeJ (C3H) mouse osteoblasts were seeded at 60,000 cells in 24-well plate for 24 hr. The cells were tranfected with the test siRNAs using the HiPerFect Transcription reagent (Qiagen). Briefly, 3 µl of HiPerfect Transcription Reagent was added to 100 µl of DMEM containing 75 ng of *Lepr* or negative control siRNA duplex. The reaction mixture was incubated for 10 min at room temp and was then added to each cell culture well containing 500 µl of fresh DMEM and 10% FBS. After 16 hr of incubation at 37^{o}C, the medium was replaced by fresh DMEM containing FBS. The effectiveness of *Lepr* suppression was assessed by Western immunoblot using an anti-Lepr antibody after an additional 24-48 hrs of incubation at 37^{o}C. The protein loading was normalized against the cellular actin level using a specific anti-actin antibody.

Fig. 4A shows that the 24-hr treatment with *Lepr* siRNA2 reduced cellular leptin receptor protein level in B6 osteoblasts by >70%. Down-regulation of *Lepr* expression in B6 osteoblasts by *Lepr* siRNA2 enhanced both basal (i.e., static control) and fluid shear-induced Erk1/2 phosphorylation much further in B6 osteoblasts (Fig. 4B). In addition, the siRNA-mediated knockdown of *Lepr* expression in C3H osteoblasts also led to restoration of their mitogenic responsiveness to the shear stress (Fig. 4C). These data support our contention that Lepr or its signaling is a negative regulatory mechanism in the context of mechanotransduction.

Specific Objective #3. To generate a colony of Lepr-deficient mice for investigation.

In order to ensure that the observation seen with leptin-deficient mice was due to the lack of Lepr signaling and not indirectly through leptin deficiency, we sought to determine the effect (or the lack of an effect) of mechanical loading on periosteal bone formation in Lepr-deficient mice. For this work, we need to establish a Lepr KO mouse colony in our laboratory for investigation. Accordingly, we have purchased a breeding pair of heterozygous Lepr KO mice (B6.Cg-mLpr^{db}/+ +/J) from the Jackson Laboratories. Homozygous Lepr KO mice are infertile and cannot be used in the breeding purpose. Because *Lepr^{db}* homozygotes are functionally sterile, the coat color marker misty (*m*) has been incorporated incorporated into stocks for maintenance of the diabetes (db) mutation. This repulsion double heterozygote (*m* +/+ *Lepr^{db}*) would facilitate identification of heterozygotes for breeding, while the coupling double heterozygote, (*m Lepr^{db}*/+ +, this strain) allows identification of homozygotes before the onset of clinical symptoms. The recessive misty mutation causes a mild dilution of coat color.

The breeding program has been initiated approximately 3 months ago. The breeding pair produced a litter approximately 1 and a half month ago. Unfortunately, the mother cannibalized all pups. The breeding pair has recently produced another litter of six pups. We will genotype the pups after weanling, and heterozygous mice will be used in further breeding to establish a colony. Homozygous pups will be used for subsequent *in vivo* and *in vitro* studies.

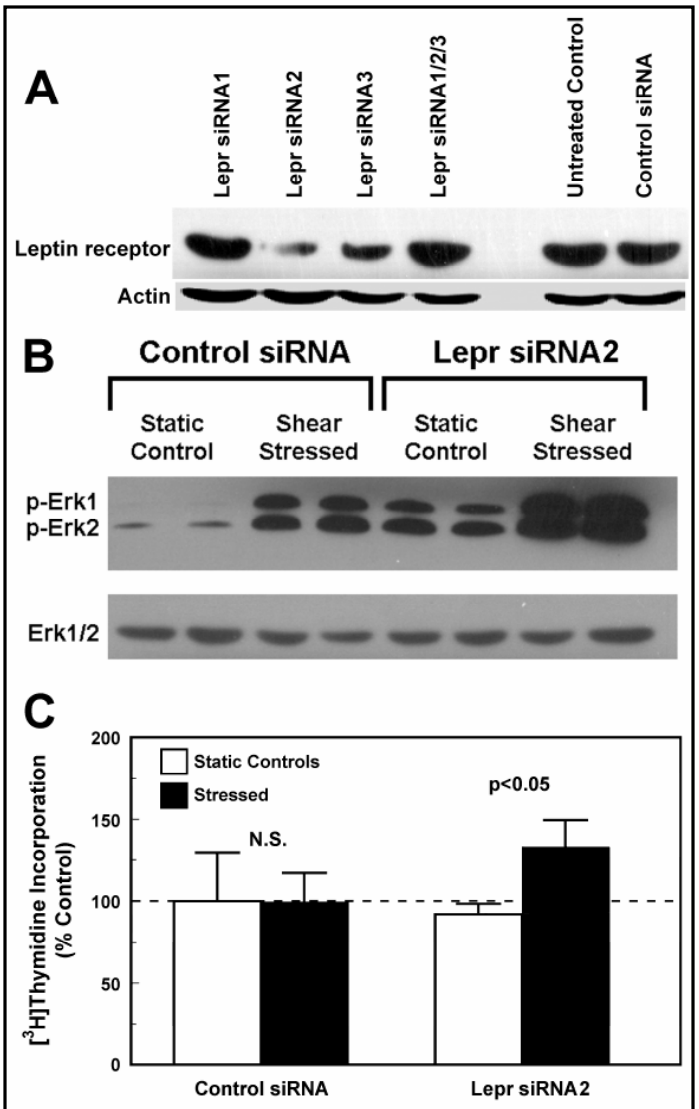

Figure 4. siRNA-mediated suppression of *Lepr* expression enhanced basal and shear stress-induced Erk1/2 phosphorylation and [^{3}H]thymidine incorporation in B6 osteoblasts and restored the mitogenic response of C3H osteoblasts to fluid shear *in vitro*. A shows the effects of the 24-hr treatment of a set of *Lepr* siRNAs (1-3) alone or in combination (at a total concentration of 5 nM) on the cellular *Lepr* protein level in B6 osteoblasts. Cellular *Lepr* protein was identified by Western blots and normalized against actin. B shows the effects of the effects of suppression of *Lepr* expression by *Lepr* siRNA2 on p-Erk1/2 levels. Total Erk1/2 was measured with an anti-pan-Erk antibody. C shows that suppression of *Lepr* expression in C3H osteoblasts by a 24-hr treatment with 5 nM *Lepr* siRNA2 restored their mitogenic response to the 30-min fluid shear stress of 20 dynes/cm^2. Cell proliferation was measured by [^{3}H]thymidine incorporation during the final 6 hrs of a 24-hr incubation. Results are shown as mean ± SEM (n=4).

<u>Specific Objective # 4. To develop a pulsatile or oscillatory fluid flow system for subsequent confirmation studies</u>.

There is a concern about the physiological relevance about findings of steady shear stress as a surrogate model of mechanical loading. Although we have obtained significant important information about the mechanical signaling process with *in vitro* cell culture model employing steady fluid shear stress in the past, we feel that it would be essential to confirm our findings with a pulsatile or oscillatory flow cell culture model. A number of pulsatile or oscullatory flow cell culture models have been used by other laboratories. We chose to adapt the system developed by Jacobs *et al*. (6), because this system requires only minor modifications from our currently employed steady flow cell culture system to be converted into an oscillating or pulsatile flow cell culture system. In this model (Fig. 5), an inline pressure transducer is attached to the flow chamber inlet. The static component of flow is provided with a Harvard syringe pump. The dynamic component of flow (that yields an oscillating or pulsatile flow) is provided by a 1 ml syringe mounted in a servohyraulic loading machine. Computer generated displacement commands are followed by the servohydraulic actuator to within 50 µm resulting in delivery of a flow profile accurate to within 1 µl at any point in time. Thick rigid walled plastic tubing is used throughout the flow delivery system to minimize dynamic compliance. The static and dynamic components of flow are summed with a Y connector and delivered to the flow chamber (Fig. 5A). A schematic of the fluid

flow circuit is shown in Fig. 5B.

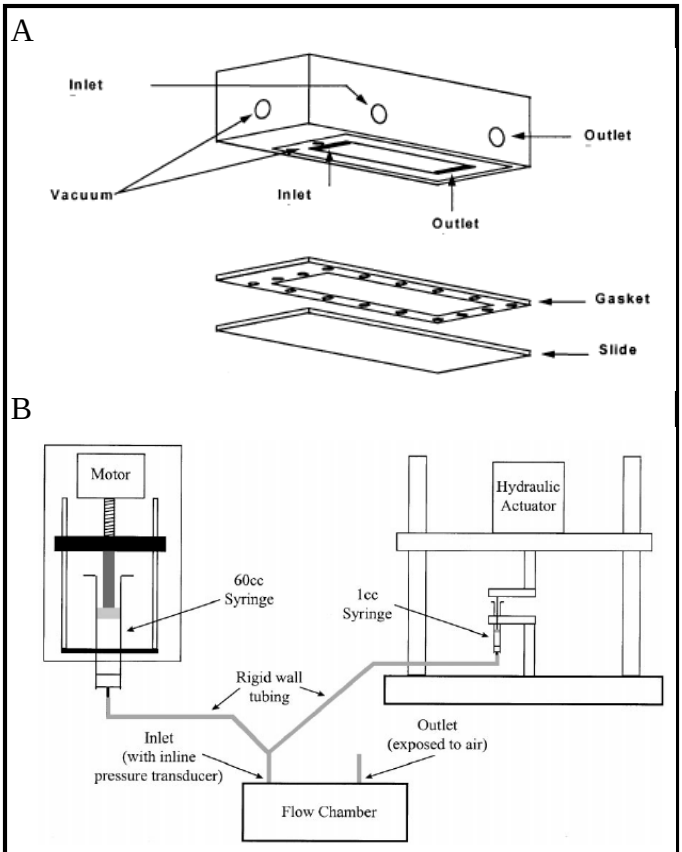

Fig. 5. A. Schematic of the parallel plate flow chamber consisting of a quartz glass slide with cells attached, silastic rubber gasket, and polycarbonate manifold. The components are held together by vacuum. The inlet and outlet ports communicate with the inlet and outlet slots. **B. A schematic of the fluid flow circuit.** Steady flow is generated by a Harvard syringe pump (upper left). Oscillating flow is supplied with an Interlaken servohydraulic loading machine (upper right). Flow from the two devices are summed with a "Y" connector and input to the flow chamber via an inline pressure transducer. If the Harvard pump alone is activated steady flow is delivered to the chamber. If only the Interlaken is activated oscillating flow is delivered to the chamber. If both are activated pulsatile flow is delivered to the chamber. Finally, the flow chamber outlet is vented to the air with a short length of tubing. These figures are adapted from Figs. 1 and 3 of ref. (6).

We are currently setting up this system and working on standardization of the flow system. Once this flow system is validated, we will use this flow system to confirm our key findings that were obtained from our past studies using the steady fluild flow system.

D. Key Research Accomplishments:

1. We have showed that leptin-deficient mice showed a significantly greater response to mechanical loading created by a four-point-bending exercise regimen, supporting our contention that the leptin-Lepr signaling acts as a negative regulatory mechanism for mechanical stimulation of bone formation.
2. We have established an effective siRNA protocol to suppress gene expression in primary mouse osteoblasts. This protocol can be used for functional testing of candidate mechanosensitvity genes.
3. We are in the process in generating a colony of Lepr deficient mice for *in vivo* and *in vitro* investigations of the regulatory role of Lepr in mechanical stimulation of bone formation.
4. We are in the process of setting up an oscillating or pulsatile fluid flow cell culture system for use in our future *in vitro* investigations.

E. Reportable Outcomes:

There is none during this reporting period.

F. Conclusion:

In summary, with the use of leptin-deficient mice *in vivo* and their osteoblasts *in vitro* using a steady flow shear system, we have now compelling *in vivo* and *in vitro* evidence that the leptin-Lepr signaling mechanism may play key regulatory role in determining the mechanosensitivity of osteoblasts. We have also established a reliable *in vitro* siRNA strategy for the application to mouse osteoblasts in conjunction with our fluid shear stress technology as a functional test for their role in mechanotransduction.

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[1142] Anabolic Response to Skeletal Loading in Mice with Targeted Disruption of Pleiotrophin Gene

C Kesavan, S Mohan.. Jerry L Pettis VA Medical Center.

Bone formation induced by mechanical loading (ML) results from increased recruitment, proliferation and differentiation of osteoblast lineage cells. Pleiotrophin (PTN), an extracellular matrix associated protein, implicated in diverse functions, is also involved in recruitment of the osteoblasts to the site of bone formation. In a recently published study using whole genome microarray approach to identify the genes and the signal pathways responsible for ML-induced bone formation, we found that PTN expression was increased by 4-fold in response to ML in a good responder C57BL/6J (B6) mice. Furthermore, transgenic overexpression of PTN in mice resulted in increased bone formation. Therefore, we sought to determine whether the anabolic effects of ML on bone formation are mediated by PTN. We first evaluated time course effects of ML on expression levels of PTN gene using real time RT-PCR in 10 week old female B6 mice. A 9N load was applied using a four-point bending device at 2Hz frequency for 36 cycles, once per day for 2, 4 and 12 days on the right tibia and the left tibia was used as internal control. Four-point bending caused an acute increase in PTN expression (2-fold) within 2 days of loading and further increased (3 to 6 fold) with continued loading (4 to 12 days). The increase in PTN expression in response to ML was also seen in 16 and 36-week old mice. Based on these findings, we next used mice with targeted disruption of PTN gene to evaluate the cause and effect relationship between the change in PTN expression and ML induced changes in bone response. Since the mechanical strain produced by a given load depends largely on bone size, we measured periosteal circumference in the tibia of knockout (KO) and control mice and found no differences (4.55±0.24 vs 4.69±0.21, p=0.50). We, therefore, applied 9N load for both groups of mice (n=6-7). Quantitative analysis of ML-induced skeletal response measured by pQCT showed that two weeks of four point bending increased vBMD and bone size by 8% and 6% respectively in the PTN KO mice compared to the 11% and 8% increases seen in the littermate control mice. Although BMD and bone size response to ML were reduced by 23% (p=0.21) and 18% (p=0.13) respectively in PTN KO mice compared to control mice, these changes were not statistically significant. The issue of whether lack of significant difference in ML response between PTN KO and control mice is due to compensation by other members of PTN family is being pursued. In conclusion, our findings using PTN KO mice seem to suggest that PTN is not a key upstream mediator of the anabolic effects of ML on the skeleton.

C. Kesavan, None.
U.S. Army

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Session Info: Concurrent Oral: Bone Biomechanics and Quality II (8:00 AM-9:30 AM)
Presentation Time: 08:45 AM
Room: 516 ABC

Lack of anabolic response to skeletal loading in mice with targeted disruption of the pleiotrophin gene

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Abstract

Background

In a previous study we showed, using the whole genome microarray approach, that pleiotrophin (PTN) expression was increased by 4-fold in response to mechanical loading (ML) in a good responder C57BL/6J (B6) mice. To address PTN role in mediating ML effects on bone formation, we first evaluated time course effects of ML on expression levels of PTN gene using real time RT-PCR in 10 week female B6 mice. A 9N load was applied using a four-point bending device at 2Hz frequency for 36 cycles, once per day for 2, 4 and 12 days on the right tibia and the left tibia was used as internal control.

Findings

Four-point bending caused an acute increase in PTN expression (2-fold) within 2 days of loading and further increased (3-6 fold) with continued loading. This increase was also seen in 16 and 36-week old mice. Based on these findings, we next used PTN knockout (KO) mice to evaluate the cause and effect relationship. Quantitative analysis showed that two weeks of ML induced changes in vBMD and bone size in the PTN KO mice (8% and 6% vs. non-loaded bones) were not significantly different from control mice (11% and 8% in vBMD and bone size vs. non-loaded bones).

Conclusion

Our results imply that PTN is not a key upstream mediator of the anabolic effects of ML on the skeleton.

Findings

Background

Mechanical loading is now recognized as an important stimulator of bone formation. Numerous studies in animal and humans, using various loading models have demonstrated that loading increases bone mass while unloading decreases bone mass [1-6]. To date, reports have shown that several growth factors and signaling pathways are known to be activated by ML [7-11]. However, the relative contribution of each of these pathways to ML induced bone formation is not known. We previously, using genome-wide microarray approach have reported that mechanical loading by four-point bending caused a 4-fold increase in Heparin binding growth factor, otherwise known as PTN, in a good responder B6 mouse [7]. PTN, a 36 amino acid bone growth factor rich in lysine and cysteine residues, is also known as Osteoblast Specific Factor 1. PTN is involved in diverse functions, which includes: cell recruitment, cell attachment and proliferation, differentiation, angiogensis, and neurogenesis [12-14]. *In vitro* studies have demonstrated that PTN has the ability to promote adhesion, migration, expansion and differentiation of human osteoprogenitor and MC3T3-E1 cells [15-17]. *In vivo* studies using transgenic approach have shown that ovariectomy induced bone loss, due to estrogen deficiency, were protected by an increase in the expression of the PTN gene [18]. Another transgenic study, with overexpression of the human PTN gene showed an increase in cortical thickness, bone volume and cancellous bone volume [15]. In addition, immunocytochemistry studies has provided visual evidence for PTN at the site of new bone formation [15, 16]. Based on the above findings and our data that PTN expression is increased in response to ML, we hypothesize that PTN play a role in mediating anabolic

effects of ML on bone formation. To test this hypothesis, we performed ML using four-point bending device on mice with disruption of PTN gene and control mice with intact PTN gene.

Methods

Mice

Female C57BL/6J (B6) mice were purchased from Jackson laboratory (Bar Harbor, ME). PTN gene knock out (KO) mice (PTN-129 in B6 background) were generated by Dr. Thomas F. Vogt and the breeding pairs were kindly provided by Princeton University, NJ, USA, for our studies. PTN KO mice were crossed with wild type B6 mice to generate the heterozygotes. These were crossed with each other to generate 25% homozygous PTN KO mice, 50% heterozygous and 25% littermate wild type mice. The body weight of PTN KO and control mice used for this study are 18.20 ± 0.95 g and 19.0 ± 1.39 g, respectively. The differences in body weight were not statistically significant ($p=0.20$). All mice were housed under the standard conditions of 14-hour light and 10-hour darkness, and had free access to food and water. The experimental protocols were in compliance with animal welfare regulations and approved by local IACUC.

Genotyping

At 3-weeks (wks) of age, DNA was extracted from tail of female mice, using a PUREGENE DNA purification kit (Gentra System, Inc., Minneapolis, MN) according to the manufacturer's protocol. Polymerase chain reaction (PCR) was performed to identify PTN KO mice from wild type or heterozygous mice. Primers specific for neomycin gene

(forward 5’ CTT GCT CCT GCC GAG AAA GTA T 3’ and reverse 5’ AGC AAT ATC ACG GGT AGC CAA C 3’ with a PCR product of 369 bp). Primers specific for PTN gene (forward 5’ TCT GAC TGT GGQA GAA TGG CAG T 3’ and reverse 5’ CTT CTT CCA GTT GCA AGG GAT C 3’ with a PCR product of 147 bp) were used for genotyping. The following conditions were used to perform the PCR reaction: 95°C for 2 minutes; 35 cycles at 95°C for 40 sec, 57°C for 40 sec, 72°C for 40 sec; 70°C for 40 sec. The PCR products were run on a 1.5% agarose gel and the image taken with a ChemiImager 4400 (Alpha Innotech Corp., San Leandro, CA).

In vivo loading model/ regimen

ML was performed using a four-point bending device [Instron, Canton, MA], as previously reported [1]. The mice were loaded using a 9.0 ± 0.2 Newton (N) force at a frequency of 2 Hz for 36 cycles, once a day under inhalable anesthesia (5% Isoflurane and 95% oxygen). The right tibia was used for loading and the left tibia as internal non-loaded control.

For the time course study, the loading was performed at 2-, 4- and 12-days on 10-week female B6 mice. After 24 hours of the last loading, mice were euthanized and tibiae were collected for RNA extraction.

For varying age groups of female B6 mice, female PTN KO and control mice, the loading was performed for 12 days. After 48 hours of the last loading, following *in vivo* bone measurement, mice were euthanized; tibiae (loaded and non-loaded) were collected and stored at -80°C for further experiments.

Peripheral quantitative computed tomography (pQCT) measurements

To measure four-point bending induced changes in the bone parameters in loaded and non-loaded tibiae, we used pQCT (Stratec XCT 960M, Norland Medical System, Ft. Atkinson, WI) as described previously [1].

RNA extraction

RNA was extracted from the loaded and non-loaded bones using qiagen lipid extraction kit [Qiagen, Valencia, CA], as previously described [1]. Quality and quantity of RNA were analyzed using the 2100 Bio-analyzer (Agilent, Palo Alto, CA, USA) and Nano-drop (Wilmington, DE).

Reverse Transcriptase - Real time PCR

Using 200ng purified total RNA, first strand cDNA was synthesized by iScript cDNA synthesis kit (BIO-RAD, CA, USA), according to the manufacturer's protocol. Quantitative real time PCR was performed, as previously described, in order to analyze the expression levels of PTN and PPIA ((peptidylprolyl isomerase A), an endogenous control) [1]. The data were analyzed using SDS software, version 2.0, and the results were exported to Microsoft Excel for further analysis. Data normalization was accomplished using the endogenous control (PPIA) to correct for variation in the RNA quality among samples. The normalized Ct values were subjected to a $2^{-\Delta\Delta}$Ct formula to calculate the fold change between the loaded and non -loaded groups. The formula and its derivations were obtained from the instrument user guide.

Statistical Analysis

Values are given as mean ± SD. ANOVA (Bonferroni's post-hoc test) and standard t-test were used to compare the difference between load and non-loaded bones at various time-points, ages and strains using the fold change and percentage data. We used Statistica software (StatSoft, Inc version 7.1, 2005) to perform the analysis and the results were considered significant at $p<0.05$.

Results and discussion

In the previous study using whole genome microarray analysis, we reported that ML caused a significant increase in PTN expression in the good responder female B6 mouse [7]. In order to confirm this finding, we evaluated temporal changes in PTN expression during 2 weeks of four-point bending. We found that ML caused a 2-fold increase in PTN expression as early as 2 days that was sustained during the entire 2 weeks of mechanical loading (Figure 1). Furthermore, ML effects on PTN expression was seen in three different age groups of mice, 10-, 16- and 36-weeks (Figure 2). These data demonstrate that PTN is a mechanoresponsive gene in the bones of mice. In contrast to this in vivo finding, an in vitro study using cultured human osteoblast cells have shown that PTN expression decreases in response to mechanical stimulation [19]. Although we cannot fully explain this discrepancy between our data and the in vitro study, possible explanations include: 1) Osteoblast responsiveness to mechanical loading may differ in vivo vs. in vitro. 2) Type of mechanical loading and the amount of strain utilized were different between the two studies.

It is well established that the amount of mechanical strain exerted by a given load is largely dependent on the cross sectional area (moment of inertia) such that a mouse with a large cross sectional area will experience lower mechanical strain and vice versa [1, 20]. In order to assure that the difference in the bone responsiveness to loading between PTN KO mice and controls is not due to difference in the mechanical strain, we measured the bone size by pQCT at tibia mid diaphysis and calculated the mechanical strain using a mathematical model (Stephen C. Cowin: Bone Mechanics Hand book, 2nd edition, 2001, chapter: Techniques from mechanics and imaging) for both sets of mice before the loading. We found that there is no significant difference in the bone size (4.55mm vs. 4.69mm, $p=0.50$) as well as in the mechanical strain for 9N (6310με vs.6351 με, $p=0.91$) between the PTN KO mice and controls. Thus, the applied load was the same for both sets of mice.

To determine if ML induced increase in PTN expression contributes to anabolic effects of ML, we performed four-point bending using a load (9N) that has been shown to exert significant changes in BMD [1]. If PTN is an important mediator of skeletal anabolic response to loading, we anticipated PTN KO mice to show reduced anabolic effects of ML on bone. We found that there was, indeed, a small reduction in ML response in the PTN KO mice (Figure 3, see additional file 1); however, these changes were not statistically significant (Since there was no difference in the response, we did not proceed any further with histomorphometeric analysis). A potential explanation for the lack of significant differences between the control and KO mice is that PTN disruption could lead to increased expression of other molecules which share similar functional properties

to compensate for the loss of PTN. For example, midkine belong to the family of HB-GAM as PTN that has been shown to have similar functional properties. Mice with midkine or PTN deficiency have been reported to have normal low auditory response while mice with both gene deficits showed impaired auditory response [21]. A similar observation has been also reported with regard to fertility. Mice with disruption of both genes were infertile while mice with deficiency in either midkine or PTN gene were able to produce similar number of offspring's [22]. Another study has shown that mouse with absence of PTN gene resulted in normal skeletal growth and this is likely due to an increase in midkine expression as evident from their microarray data [23]. Overall, these findings suggest that factors of the same family are exhibiting overlapping function and thus, interfering with the activity of one factor may not necessarily lead to disruption of physiological activities such as bone formation response to loading. The issue of whether disruption of both PTN and midkine will exert a greater deficit in the skeletal anabolic response to ML compared to individual knock out requires further study.

Additional file 1.
Format: Doc Size: 21KB
Title: pQCT measurement of bone parameters.
Description: The data in this file shows absolute changes in bone parameters in response to loading between PTNKO and control mice.

Competing interests

The author declares that they have no competing interests.

Authors' contributions

All experimental procedures, data analysis and study coordination were carried out by CK. SM contributed to the design, data interpretation and manuscript preparation. All authors read and approved the final manuscript.

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Figures

Figure 1 - Expression levels of PTN gene as a function of duration of loading.
The y-axis represents fold increase in the PTN gene in response to four-point bending on tibia and the x-axis represents duration of loading on 10-week female B6 mice. Values are mentioned as mean ± SD, [a]$p<0.01$ vs. non-loaded tibiae, [b]$p<0.05$ vs. 2-days (Post Hoc test, ANOVA), N=5.

Figure 2 - Expression levels of PTN gene as a function of age.
The y-axis represents fold increase in the PTN gene in response to 12 days of four-point bending on tibia and the x-axis represents varying age groups of female B6 mice. Values are mentioned as mean ± SD. [a]$p<0.05$ vs. non-loaded bones, N=5.

Figure 3 - Changes in bone parameters in response to ML on 10-wk female PTN KO and control mice.
Values are mentioned as mean ± SD. The y-axis represents percent increase in bone parameters in response to four-point bending on tibia and x-axis represent skeletal parameters. TA, Total area; BMC, bone mineral content; PC, periosteal circumference; EC, endosteal circumference; Cth, cortical thickness and BMD, bone mineral density. *$p<0.05$ vs. corresponding non-externally loaded tibiae, N=7.

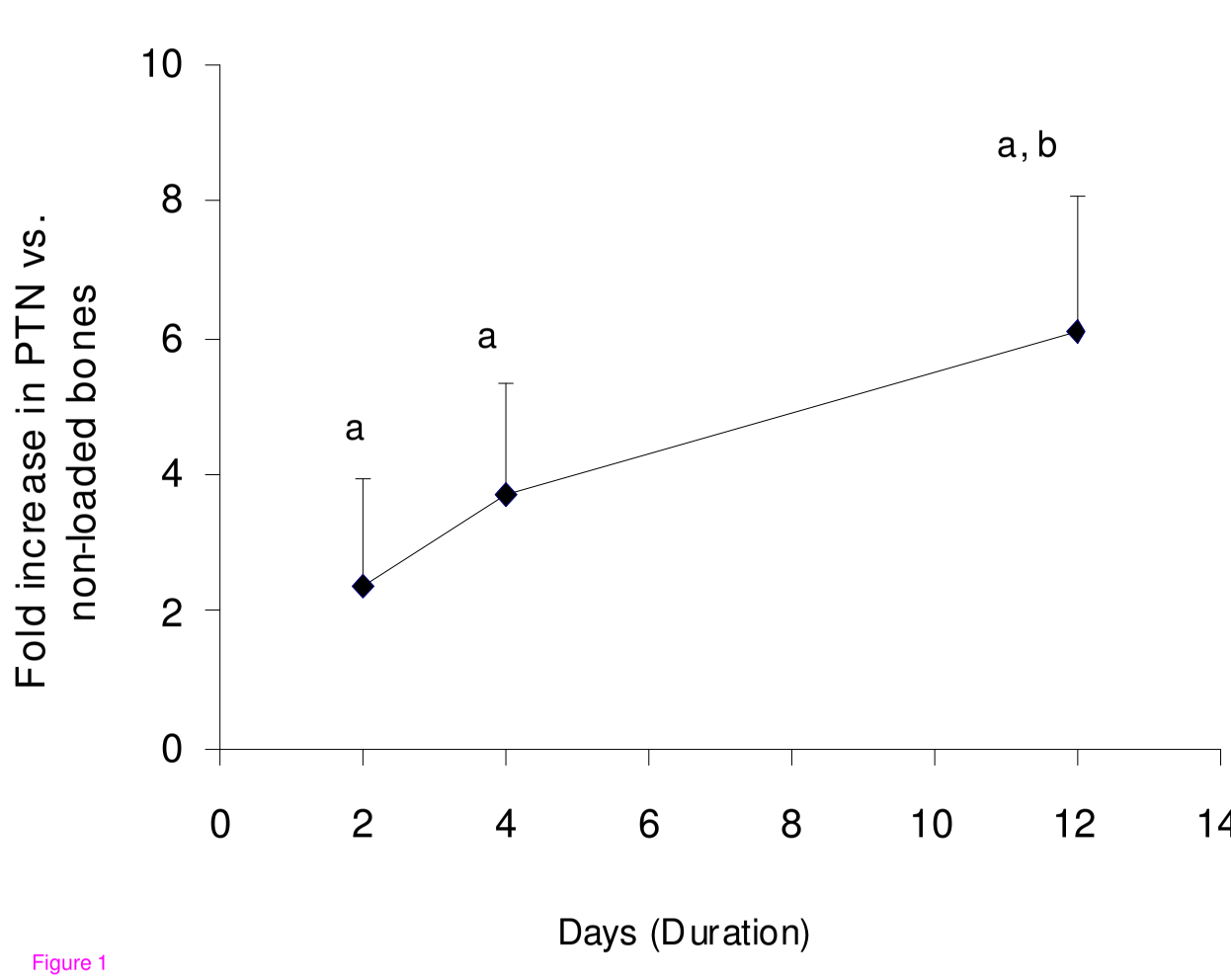
Fold increase in PTN vs. non-loaded bones
10
8
6
4
2
0
0
2
4
6
8
10
12
14
a
a
a, b
Days (Duration)

Figure 1

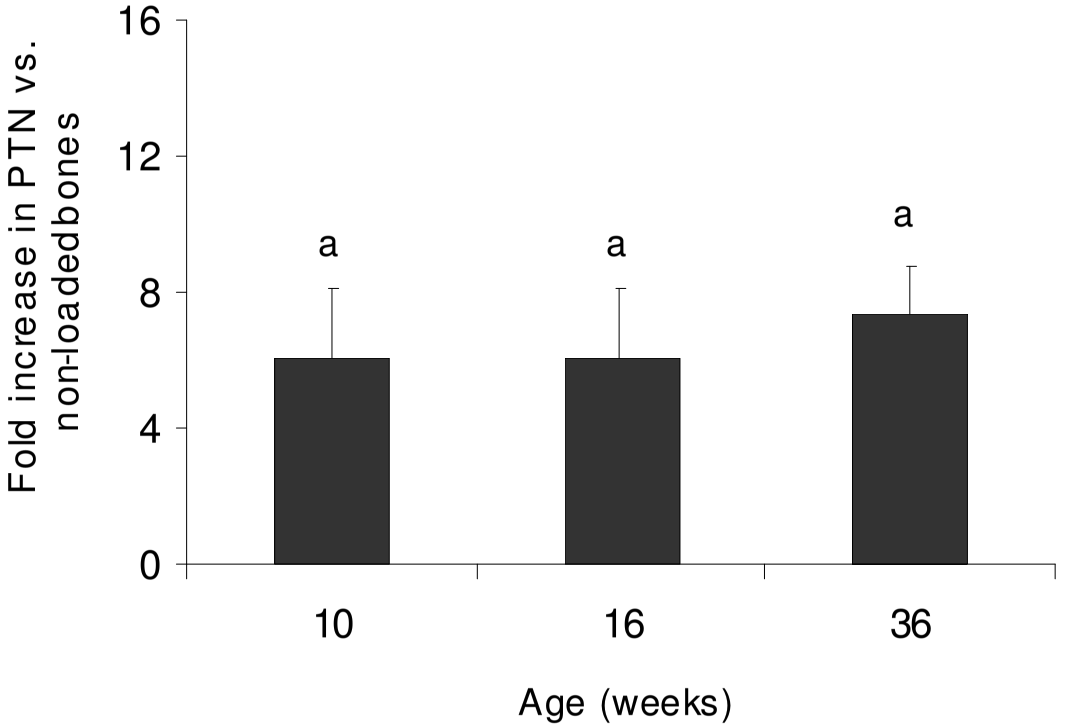
Fold increase in PTN vs. non-loaded bones
16
12
8
4
0
a
a
a
10
16
36
Age (weeks)

Figure 2

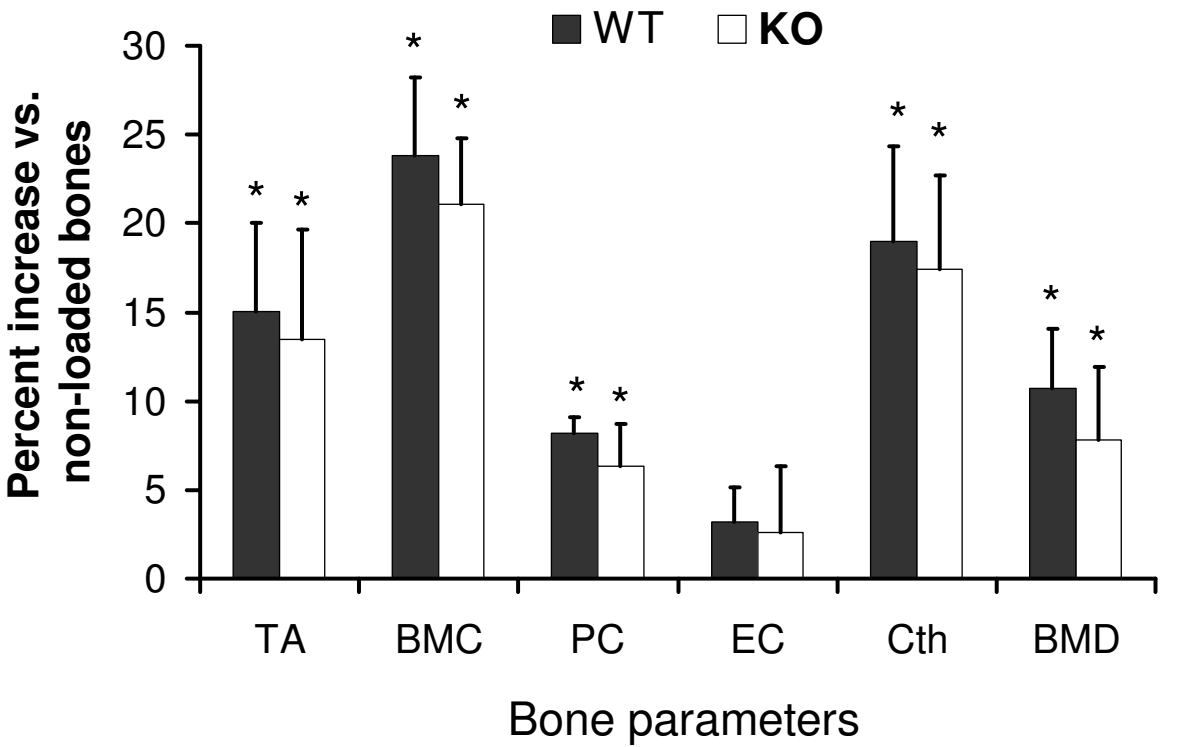
WT
KO
Percent increase vs. non-loaded bones
30
25
20
15
10
5
0
*
TA
BMC
PC
EC
Cth
BMD
Bone parameters

Figure 3

Additional files provided with this submission:

Additional file 1: additional file 1.doc, 29K
http://www.biomedcentral.com/imedia/9779509782385470/supp1.doc

T151

The Increased vBMD and Bone Area due to Mechanical Loading Gradually Decreased Following Cessation of Loading.

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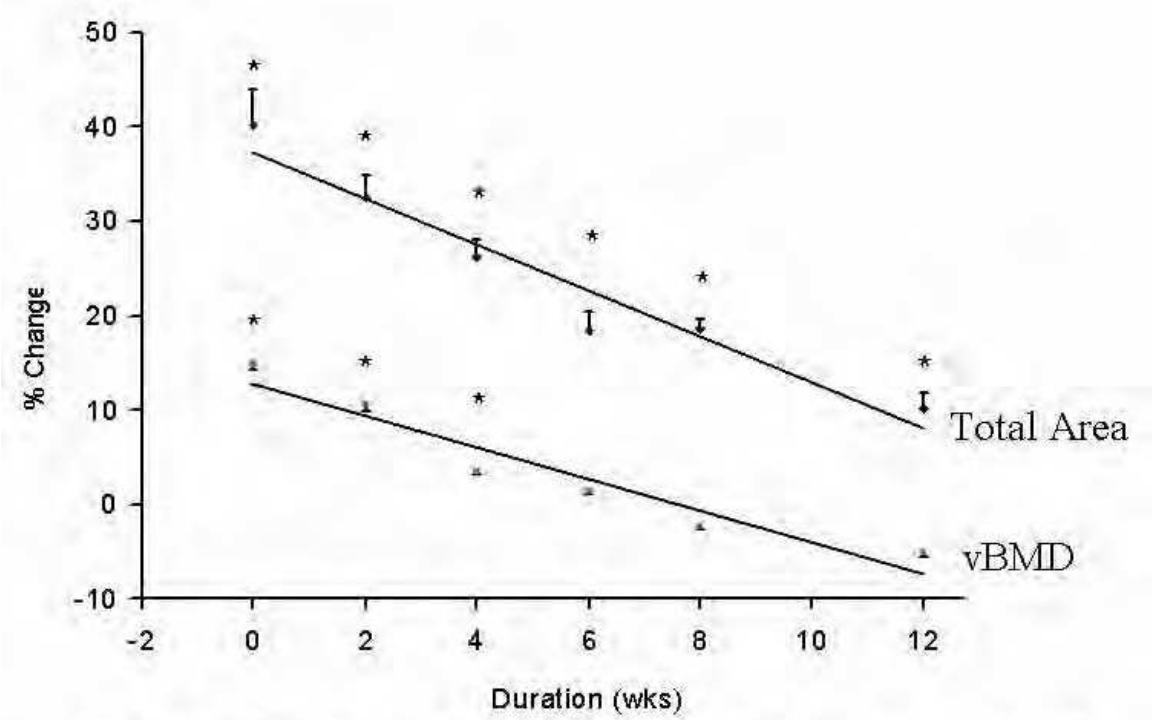
Dynamic loads lead to increases in volumetric (v) BMD and cross sectional area. However, the issue of how long these gains are maintained after cessation of loading has not been fully understood. To address this question, we performed a long term study in which skeletal changes were monitored every 2-4 weeks for a 12 week period following cessation of external loading. A four-point loading device was used to apply external loading (9N at a frequency of 2Hz for 36 cycles once per day for 12 days) to the right tibia of 10-week old female mice. Skeletal changes were monitored by in vivo pQCT. The value of the left non-loaded tibia was subtracted from the value of the right loaded tibia to determine the changes associated with loading at eachtime point. Two-weeks of four-point loading caused a drastic 40% increase in total area (TA) and 15% increase in total vBMD. However, the increase in size due to external loading did not continue once the external loading was stopped. Furthermore, cessation of loading resulted in a continuous loss of both bone size (TA) and vBMD. The vBMD returned to normal at 12 weekswith a half life of 6 weeks. The TA declined with a half life of 8.5 weeks and was still significantly elevated at 12 weeks. Thus, the decline in elevated TA proceeded at a much slower pace than the loading induced increases in vBMD. Conclusions: 1) External loading-induced increases in bone are lost over a period of time but at a much slower pace than the induction of the increases; 2) While a single burst of external loading provides increased bone mass temporarily, periodic loading may be necessary to maintain long term bone strength.



Values are given as mean ± SE, *p<0.05 vs. non-loaded bones, n = 5